

July could yet be a fateful month

Wonder at the way in which the world has changed in the past year should not blind us to the importance of the month that lies ahead. When that is over, everything may again be different.

FROM this weekend, the planned monetary union of the Federal Republic of Germany (West Germany) and the German Democratic Republic (East Germany) will take effect, although the continuing basis of German security remains to be determined. Next week, Mr Mikhail Gorbachev will face a divided congress of the Soviet Communist Party (of which he is the general secretary). By then (5–7 July) the governments of the North Atlantic Treaty Organization (NATO) will have met in London. By 9 July, the heads of government of the seven principal industrialized states will be meeting in Houston, Texas. Some of their foreign secretaries will be back in Paris on 17 July for the next meeting of the '4 + 2' commission trying to hammer out an East–West understanding on the future of Germany. Diplomats are no doubt relieved that the crucial meeting of the Conference on Security and Cooperation in Europe (CSCE) on the European question has been postponed until October. Yet, despite the good luck that there are so many opportunities for decision at such a crucial time, events are moving even faster. Action may get the better of good intentions, of which there are too few.

European security and the future of the Soviet Union are the overriding questions, as they have been for the past year. Everybody is to blame that the first remains as obdurate an issue as it is. From the outset of the transformation of Eastern Europe with the Polish elections more than a year ago, it has been plain that NATO had an interest in common with the Soviet Union in providing an assurance that it would not take military advantage of the developing situation. The reason is plain: without such an assurance, the pace of change in the heartland of the Soviet Union and in its republics will be inhibited. But while important people have made the right noises, there has not yet been a tangible change in the form of NATO's military arrangements — except that the United States has administratively abandoned plans for modernizing its short-range nuclear missiles based in Europe.

Appraisal

What NATO should now be undertaking is an appraisal of military Europe as the Soviet Union sees it — the view 'from over the hill' as the strategists say. Poland, Czechoslovakia and Hungary, in their different ways, are heading towards being Western-style economies, and in varying degrees resent the fact that Soviet military forces are still stationed there (as they will be in East Germany for a

still undetermined period). Between them, the four territories are a buffer zone on which the Soviet Union has counted to forestall an invasion along the lines of that in 1941. How can the Soviet Union be helped to believe that changed political arrangements have not diminished that security? NATO (like the territories concerned) has a further interest in arranging that future Soviet governments are not tempted to seek militarily to reverse the past year's changes. With all four territories drawn towards the West by trade and investment, the zone will seem from the East to be less secure.

Profit

NATO members could only profit if the buffer zone seemed more substantial in Soviet eyes. How can that be arranged? The announcement (by the Soviet Union as well as NATO) that short-range nuclear weapons are being partly withdrawn from central Europe should help, but is insufficient. An agreement on conventional forces in Europe would go further, but its attainment has been complicated by the developments of the past year. It has not been sufficiently appreciated that these same changes have altered the role of Soviet forces stationed in the buffer states; their wider function is now more like that of US forces in the West, whose presence is the tangible foundation of the transatlantic bridge. The chief difference is that at least some of the governments concerned are unwilling hosts. Although the CSCE (otherwise Helsinki) treaty requires its signatories, which include the Soviet Union and the United States, to respect existing European frontiers, the sanction against transgression will presumably be the threat of nuclear war.

Broadly speaking, there are two patterns that might be followed in the immediate future, one of which is an extrapolation of the status quo, no doubt accompanied by an agreement between NATO and the Soviet Union on the nature and strength of forces in the West and East respectively. The other, more radical, is deliberately to broaden the buffer zone by an agreement (there would have to be a negotiation) that foreign troops should not be stationed in the traditional buffer zone or even in what is now West Germany. That would go against the grain for many Western governments, and perhaps even in the new Germany, but there would be advantages for both sides that should not be overlooked.

The buffer zone, seen from the East, would be evidently more substantial, but so it would become from the other



side. In military terms, NATO's much-discussed plan for dealing with incursions by the use of nuclear weapons against tactical targets is predicated on the narrowness of the north German plain, but if that were broadened by the extent of East Germany and Poland, the case for battlefield nuclear weapons would be diminished almost to vanishing point. None of that, of course, would be inconsistent with continued German membership of NATO, while the strategic threat of nuclear war if vital interests were undermined would persist. But, for NATO members, the important prize would be to rid the buffer states of Soviet forces, with the threat to the natural development of the states concerned their continued presence might in future bring.

That prize would be worth a lot. For geographical reasons, NATO could afford it only if France were to play a fuller part in NATO. (As a stopgap, there is something to be said for dusting off the Rapazki plan for a denuclearized zone in just this territory advocated by the then Polish prime minister in the 1950s.) But such a more radical transformation of East-West military arrangements in Europe would also assist the process of reform in the Soviet Union, not merely by liberating economic resources, but by providing a framework for further development of European security arrangements. Soviet interests would, for example, be less directly threatened by Lithuanian independence if it were no longer necessary to keep supply lines with Poland intact.

This, in short, would be one way of answering the question, taken up this week at the Dublin meeting of the European Communities and likely also to dominate the meeting at Houston, of "How to help Mr Gorbachev?". The question, as stated, is over-simple. Gorbachev is an intelligent and courageous man whose plans for reform may yet be defeated by what is now apparent — that the social structure in the Soviet Union has hitherto been maintained by the Communist Party, not the government. Now that the Party's authority has been lost, everything is chaos. The new Supreme Soviet has the makings of an effective parliament, but its electoral legitimacy remains to be established. Meanwhile, there are 250 million people to feed, while the winter is only a few months away. The urgent need is for Western credit — lots of it. West Germany was talking of \$20,00 million just a week ago, but that is a tiny fraction of the investment that will be needed if the Soviet Union is to be made prosperous and, thus, stable. It is not just that Soviet enterprises function badly, but that even if they functioned well, they would most often be producing goods and services that people no longer want. Ultimately, new investment on a scale 100 times greater will be needed, most of it from Soviet savings. What the West could do is to demonstrate by example that money on such a scale can be put to good use. It would be unusual, but not unreasonable, if funds for investment were tied by the condition that they should be managed by Western companies. But can the Houston summit get word of some such scheme to Moscow before the Party congress is over? □

PhD by dissection

Are literary studies running out of thesis topics? And are the natural sciences as adventurous as they might be?

EVERYBODY, of course, knows what a PhD thesis is: the report by a beginning researcher of an original piece of work. PhD supervisors know that there is no shortage of problems. Their skill is to help a student specify a problem that may be tractable in a reasonable time and which, if solved, will prove interesting. The essence of the exercise is that a student should, at some stage, be confronted with the need on his or her own to make sense of what is not yet known. But is it always like that?

Émile Lavernay, emeritus professor at the University of Nice, on Saturday startled more than a hundred academic members of what he called the 'D.H. Lawrence industry' gathered at Montpellier with a teasing question about the institution of the PhD in English literature ('Eng.Lit.' for short): if the numbers of graduate students are increasing more quickly than the number of writers whose works are judged worthy of critical appraisal, is there not a danger that well-known literary works will be dissected in ever-finer detail, with the consequence that the theses that result are of interest only to their authors and the supervisors thereof?

It is an important question, even if over-simplified, with whose implications those who work in other fields should at least sympathize. The study of literature is valuable, and those who say that they cannot see what there is in Eng. Lit. (or other species of Lit.) that they do not themselves practise when they have the leisure to read a novel are true philistines. Its purpose is to make literature intelligible, ultimately so as to benefit readers. Its methods range from the verification of texts and the analysis of verbal patterns to the search for allusions (in one text to another) and social influences (on, or even, by particular authors), but the core of this activity is literary criticism — the definition and the appraisal of qualities of texts. It is not so much a question of whether D. H. Lawrence's *Sons and Lovers* is a good novel, but of why, with all its faults, it is so regarded.

The essence of Lavernay's complaint is that theses pretending to be original criticism of an author or work must inevitably be less and less illuminating. He might have added that there is a danger that the process can at once trivialize an important work and give trivialities undue importance, thus impeding enlightenment. The trouble is that so long as only excellent literature qualifies for this attention, the demand for thesis topics can be met only by slicing old topics more thinly. Natural scientists will — certainly they should — naturally be sympathetic, knowing as they do that the process of discovery necessarily raises more questions than it answers. But they should also be careful not to patronize the Eng. Lit. fraternity, at least until they can be sure that their own PhD thesis topics are not also salami slices. □

Protests oust science at AIDS conference

- US immigration policy lambasted
- Activists take to the podium

San Francisco

THE politics of the AIDS epidemic and the growing involvement of AIDS activists in determining the course of clinical trials of new drugs dominated discussion at the Sixth International Conference on AIDS which ended in San Francisco last week. Few major scientific announcements were made, strengthening suspicions that the days of the conference as a forum for presenting new research are numbered.

Politics were apparent at the opening ceremony in a display of anger over government policy that makes it hard for people infected with the HIV virus to obtain visas to enter the United States — a policy which caused almost 100 organizations to boycott the conference. Led by Peter Staley, a representative of the AIDS activist group ACT-UP, thousands of delegates stood and chanted "300,000 dead from AIDS — where is George?"

Unlike the heads of state of countries that had hosted the conference in previous years, US President George Bush declined to appear at the opening ceremony. Instead he was to be found attending a fund-raising event for Senator Jesse Helms who — as it happens — sponsored the offending amendment barring entry to anyone infected with HIV.

Although events inside the conference centre hinted at a new era of cooperation between researchers and AIDS activists, outside, demonstrations by those frustrated by the failure of government to stem the AIDS epidemic continued throughout the week. A particular target was the bias against the inclusion of women in clinical trials (see next page). Arrests were made as groups of protesters attempted to block traffic near the conference centre.

Inside the centre, the only major disturbance greeted the closing speech from Department of Health and Human Services Secretary Louis Sullivan. Seen by activists as a representative of the White House, his speech was drowned by whistles and chants. Earlier, a satellite session chaired by Jerome Groopman of Harvard University Medical School had to be postponed when it was invaded by protesters. Otherwise the conference was a testament to the organizers' efforts to integrate activists by giving them speaking slots.

The involvement of groups such as ACT-UP also reflects a growing willingness of researchers to listen to activist arguments about priorities in AIDS research. Addressing delegates at a session organized by activists, ACT-UP's

spokesman Mark Harrington spoke of his "partnership" with Anthony Fauci, director of the National Institute of Allergy and Infectious Disease, who has been heavily involved in coordinating the clinical trials of AIDS drugs; Fauci has promised ACT-UP places on a research advisory committee.

Many scientists are also willing to acknowledge the role of activists in speeding the process of drug approval by the US Food and Drug Administration and in persuading them to endorse a "parallel track" scheme whereby HIV-positive individuals and patients with AIDS who are not eligible to enroll in clinical trials can obtain access to experimental drugs.

ACT-UP released its new agenda at San Francisco, giving an account of how clinical trials could be speeded up further and how the collection of data on experimental drugs could be facilitated using a "middle track" scheme. Whereas "parallel track" simply distributes experimental drugs to patients as soon as they clear preliminary safety tests, "middle track" would enable manufacturers to gather a limited amount of data on a drug's toxicity and effectiveness at different doses by allowing them to monitor the progress of individuals not enrolled in the official clinical trial.

Conflicts over the key question of how to balance careful science with the patient's need for rapid treatment did not vanish altogether. A heated debate erupted at a plenary session on clinical trials when Martin Delaney of Project Inform, a group that advocates unofficial community-based research, unveiled the startling, but sketchy, results of an "underground" trial — completed in record time — of Compound Q, the most controversial of all experimental AIDS drugs.

Delaney's results, obtained in collaboration with San Francisco physician Larry Waites, show apparent dramatic rises in the T4 cell counts of 8 out of 46 patients after treatment with the drug. In the panel discussion that followed, Delaney's talk, acclaimed as a triumph by activists, met with a barrage of criticism. First with his gloves off was Arnold Relman, editor of the *New England Journal of Medicine*, who accused Delaney's organization of practising "black magic".

Criticizing Delaney for not releasing detailed data on the 46 patients, Relman called him "irresponsible" for "making such claims without publishing your data"

A second agent?

THE most controversial announcement of the conference came from Luc Montagnier, leader of the French group that first discovered the AIDS virus at the Institut Pasteur, who claimed that he had isolated a new infectious agent associated with the development of AIDS.

The agent is a mycoplasma, a small bacterium-like organism, which Montagnier says may enhance the cell-killing properties of the HIV virus, thus quickening the decline of the immune system. Montagnier's claim is based on his group's isolation of mycoplasmas from the blood of AIDS patients put together with observations that coinfection with mycoplasmas of HIV-infected cells in culture increases virus proliferation. Addition of an antibiotic that kills mycoplasmas slows virus production, suggesting, says Montagnier, that certain antibiotics may have some effect in slowing the progress of AIDS. His group has already begun a small clinical trial.

Montagnier's claims were met with scepticism. In a question-and-answer session he was asked for more details about the way in which mycoplasmas accelerate virus proliferation and whether he could be sure the blood samples had not become contaminated with mycoplasma in the laboratory. Montagnier argued strongly against his results being purely an artefact of laboratory contamination but agreed that he could not rule out that the effect of mycoplasmas on virus production came through some non-specific action, such as stimulation of cytokine production.

D.C.

or subjecting them to peer review. James Kahn, who is supervising government trials of Compound Q at San Francisco General Hospital, advised caution in interpreting the data, noting that two patients died last year after treatment with Compound Q.

At a press conference following the session, Daniel Hoth, director of the NIH's AIDS research effort, emphasized the need to move as quickly as possible with clinical trials, but cautioned of the dangers of following "*drug de jour*" fads. But, regardless of the scientific merit of the Compound Q study, its description at the conference marked a turning point in the debate over when people suffering from a disease should have a say in a research agenda intended to help them.

That debate may not be completed in the United States. Harvard University, chosen for the conference in 1992, will withdraw its support if immigration policy is not changed. The International AIDS Society which sponsors the conference has reportedly given Congress until this autumn to repeal the law or find the conference cancelled.

David Concar

It's still a man's world

Washington

DESPITE four years of policy promoting balanced representation of women in clinical trials funded by the National Institutes of Health (NIH), many studies are still biased towards men, according to a General Accounting Office (GAO) report released last week. The issue was dramatized at the Sixth International Conference on AIDS (see page 753) when women protesters blocked traffic in central San Francisco to draw attention to the lack of clinical research on women who have AIDS.

"American women have been put at risk by medical research practice that fail to include women in research studies," said Patricia Schroeder (Democrat, Colorado) at a congressional hearing on the report last week. It is not known if aspirin can help to prevent coronary disease in women because the study establishing aspirin's value looked at 22,071 men, but no women, said Representative Olympia Snowe (Republican, Maine).

Iris Schneider, co-chair of the NIH advisory committee on women's health issues and assistant director for programme operations and planning at the National Cancer Institute, says that scientists sometimes avoid including women in their childbearing years because of concern that the treatment may affect fertility.

A fear that results will be distorted because of hormonal changes during menstruation has also biased some trials, Schneider says. "That is not something I can accept. Just because someone says that [women in clinical trials] presents a problem, that doesn't mean you can just walk away from it", she says. "There are clearly some specific cases in which there was a problem, but how large the problem is, no one knows". NIH is trying to obtain better statistics.

GAO, in its investigation, reviewed 50 recent applications for NIH grants. Twenty per cent of the applications did not discuss the sex of the study population. Over one third of the proposals indicated that both sexes would be included but did not say in what proportions. Some of the studies using only men gave no explanation of why that choice had been made.

Although Schneider's NIH committee recommended publication of a policy on women in 1986, it was not published in the guide for outside researchers applying for NIH grants until 1989.

And NIH staff have yet to be trained about what the policy means and how it should be carried out, she says. Following two internal surveys that indicated that sexual imbalances existed in research within NIH, Schneider's committee sent new recommendations to former NIH director James Wyngaarden shortly

before he left NIH last year. But it has yet to be acted on, she says.

The GAO report recommends that NIH train its staff and reviewers to watch for sexual bias in clinical trials. NIH acting director William Raub promised the congressional committee that NIH would follow GAO's advice. Schneider's committee will meet on 11 July to review the GAO report, perhaps making further suggestions. And the Congressional Caucus on Women's Issues will introduce legislation next month to bar the arbitrary exclusion of women from federally funded research and to create an office within NIH to coordinate women's health research and monitor other research for sexual equality.

Shigeko Segawa

GLOBAL WARMING

Japan biding its time

Washington

UNDER the impressive title 'Action Program to Arrest Global Warming', Japan last week announced its first plan to restrict future greenhouse gas emissions. But the Council of Ministers' plan, which sets no targets beyond a vague commitment to stabilizing emissions by 2000, may be far too sketchy to lessen criticism of Japan's global-warming policy.

Compared to the recent commitment by West Germany to cut carbon dioxide emissions by 25 per cent by 2005, the Japanese plan appears remarkably non-committal. The plan states that a "concrete target should be established after a full examination in order to achieve the stabilization of greenhouse gas emissions at their lowest level by the year 2000". But it does not specify what that level might be. Japanese government agencies are still debating how quickly carbon dioxide emission should be allowed to rise over the next decade, a decision that will help determine at what level to set the post-2000 ceiling.

"According to the long energy vision by Ministry of International Trade and Industry (MITI), by the year 2000, carbon dioxide emission will increase 16 per cent. But we are trying to keep carbon dioxide emissions as close to 1990 level as possible", says Kazuo Matsushita, an Environmental Agency officer. But although MITI officials commend the Environmental Agency for its optimistic vision, they are more pragmatic in outlook. MITI will not release a concrete plan until it is sure it can stick to it, and long negotiations between the Japanese ministries remain before even that is possible.

Shigeko Segawa

Latest deforestation figures

São Paulo

New figures for the extent of Amazonian deforestation were released by José Goldemberg, Secretary of State for Science and Technology, at a meeting of Latin American remote-sensing groups in Manaus last Sunday. According to the estimates prepared by Brazil's National Institute of Space Research (INPE) from 167 Landsat images taken during the latter half of 1989, the deforested area of the Legal Amazon has now reached 404,000 square kilometres (including ancient deforestation), up from the 1988 estimate of 343,975 square kilometres.

INPE officials refuse to issue the figures as percentages because of previous controversy over what land area constitutes the Amazon (see *Nature* 339, 531; 11 May 1990). If the Legal Amazon (an administrative definition) is taken as 'the Amazon', then 8.2 per cent may be calculated to have been destroyed since colonization began in the sixteenth century; if the area of rain forest alone is considered, then 10.9 per cent has been destroyed.

The measured increase in the area of destruction over the previous year is not all real, but is in part old destruction being measured for the first time. INPE sources estimate that about 30,000 sq km of destruction is new, putting rain forest destruction at 0.8 per cent a year. That figure is down from estimates from other remote-sensing projects of 80,000 sq km of destruction for 1987 (2.1 per cent a year) and 48,000 sq km for 1988 (1.3 per cent).

Monkey business

Two Brazilian researchers have found a new species of monkey, but not deep in the Amazonian jungle where everyone might expect. The new species of lion tamarin was found in the Atlantic forest, in an island near the border of the states of Pará and São Paulo. Such coastal regions are the most populated and extensively deforested in Brazil.

The discovery was reported in a workshop last week in Belo Horizonte and the new species christened *Leontopithecus caissara*. It joins three other known tamarin species, all of which are in risk of extinction as their habitats vanish.

The new tamarin was found by two researchers from a natural history museum in Curitiba, Paraná state capital, the Museu de História Natural Capão de Embuia. Vanessa Guerra Persson and Maria Lúcia Lorini have been studying tamarins for about five years. They acted after reading a description written in 1850 of monkeys living in the region. In February this year they were rewarded by a first sighting of five tamarins.

Ricardo Bonalume

ANIMAL WELFARE: USA

Politics force new US rules

Washington

As Britain debates how to pay for its new animal welfare regulations, five years' work towards similar regulations in the United States may have been lost in another battle over economics. Under fire from the White House (see *Nature* **344**, 804; 26 April 1990), the US Department of Agriculture (USDA) has agreed to drop proposed rules that critics claimed would cost US animal facilities nearly \$900 million and propose new less strict rules next month. But Congress and activists promise a fight before any watered-down regulations become law.

Although most of the proposed US rules were weaker than their British counterparts, the few exceptions — specific standards for the exercise and well-being of dogs and non-human primates — bogged down the regulations in bureaucratic limbo while the British rules became law. As a result, USDA officials have been forced in the last month to revise the rules to stress the 'performance' of research dogs and primates, rather than the exact details of their care. Animals can be kept healthy and happy in more than one way, argued the White House and animal facility lobbyists. Demanding that facilities conform to rigid 'engineering' standards only imposes unnecessary costs, they said.

To back its claim that the proposed USDA regulations would bankrupt US research, the National Association for Biomedical Research (NABR) estimated that bringing US animal facilities up to the proposed cage and exercise standards would cost an initial \$899 million and add another \$106 million to annual costs. Almost all the expense lies in requirements that primates and dogs be given specific amounts of time and room in which to socialize and exercise, says NABR vice-president Barbara Rich.

The proposed USDA regulations went beyond current NIH guidelines for socialization and exercise by setting absolute standards. Dogs were required to be either housed in cages large enough for them to run or to be exercised for 30 minutes a day.

Individually housed primates were to be allowed out for at least four hours of exercise and social contact a week. Many animal facilities would have been forced to enlarge their animal areas substantially and hire additional handlers to meet those standards, says Rich.

Critics of the proposed rules had also argued that, by essentially codifying the current NIH guidelines for guinea pig, rabbit and hamsters cages, USDA would have forced facilities to replace existing cages with new enclosures as little as half an inch larger. USDA intends to side step

that criticism by including a 'grandfather' clause in its new rules, sources say. Animal facilities will be allowed to retain their existing cages, substituting them with larger sizes only when the old cages fall due for replacement in the normal way.

USDA officials are troubled by the new emphasis on 'performance'. Without numbers to check against, inspectors will find it difficult to verify cases of improper care, they say. And even when animal behaviour does point to negligence, inspectors will be hard pressed to defend their appraisals without quantitative evidence to back them up.

Performance-based standards "will

ANIMAL WELFARE: UK

Counting the cost of UK law

London

BRITISH universities fear that drastic cut-backs in animal research will prove necessary if the government does not provide an extra £75 million to bring university animal houses up to the improved standards required by recent legislation. David Franks, secretary of the University of Cambridge's School of Biological Sciences, says that access to animal facilities could become a serious limiting factor for research.

A new code of practice governing the care and housing of animals was introduced last year as required under the 1986 Animals (Scientific Procedures) Act. But the cost of complying with the new code became apparent only recently, after the Universities Funding Council (UFC) asked universities to bid for funds to upgrade animal houses. The code of practice specifies standards for cage sizes, air conditioning and humidity in animal houses. Although it is not a set of mandatory requirements, the Home Office says that if facilities fall well short of standards, improvements must be made over "a not too extended" time.

The universities' estimates are unlikely to be accepted in full by UFC, which says that a panel of academics will be asked to assess the bids for funds in the coming months.

One possibility is that the UFC will suggest economies by concentrating animal houses on fewer sites at each university. But the universities argue that this has already been done, and research will suffer if extra money is not found. "We're getting to the point where we can't rationalize any more", says one senior veterinary scientist, whose own institution faces a £7 million bill. Franks says that Cambridge, which is asking for £9 million, will face "serious difficulties" if the issue is

take much more effort to enforce", says Richard Crawford, director of the USDA animal care staff. "I'm not sure how it's going to work."

Animal welfare lobbyists say that if the USDA rules finally emerge in performance-based — rather than engineering-based — form, a lawsuit claiming distortion of congressional intent is likely. In a May letter to USDA, Congressman George Brown (Democrat, California), who heads the House of Representatives subcommittee that oversees USDA operations, warned that minimum standards are necessary to guarantee adherence. To further pressure USDA, one Brown aide says, "we could do all sorts of things, from holding oversight hearings to playing around with their funding".

G. Christopher Anderson

not resolved within the next year.

The current situation angers many university researchers, who point to Home Office minister David Mellor's assurance, before the 1986 Act, that the cost of changes in legislation would not fall on the universities. But the Department of Education and Science (DES) now says that this only referred to the small administrative costs linked to the new system of licensing for experimental work. A DES spokesman dismisses the universities' upgrading estimates as "speculation".

Some university administrators now say that the costs of adherence to the new standards should have been estimated when they were drafted, and the code of practice amended to give a compromise between the desirable and the affordable, similar to the current US approach (see above). Standards for air conditioning and humidity are thought by some to be too strict.

Commercial laboratories, with greater funds at their disposal, had few problems meeting the required standards. The head of the animal health division at one of Britain's largest drug companies says that his company has a constant programme of upgrading its animal facilities, so the short-term impact of the new code of practice is very slight.

A Home Office spokesman says that universities financial difficulties will not make them a special case, and enforced closure of some animal houses is a possibility if standards cannot be improved. One veterinary scientist, who is a strong supporter of the code of practice, says half of university animal houses may be below the required standard. If the majority of the British public supports the use of animals in biomedical research, he says, then "society has to be prepared to pay".

Peter Aldhous

Women and children first

Lübeck, West Germany

A TEN-YEAR programme to support young researchers in West Germany was approved in mid-June by the centre-right coalition government in Bonn and is likely to provide a new direction for research and teaching at universities far into the future. The programme will provide a ten-year increase in the number of temporary research and teaching positions at overcrowded West German universities in anticipation of a wave of retirements and will greatly expand research staff in many laboratories. There will be special emphasis on bringing more women into academic research and teaching, where they are badly underrepresented.

The programme, originally announced by the ambitious West German Education Minister Jürgen Möllemann more than a year ago, was not approved until the last minute because of the rapidly changing situation in Germany and worries over the cost of unification. Only a part of the budget has so far been approved — just DM4,000 million (\$2,400 million) of the DM6,000 million originally requested by Möllemann — leading Hubert Markl, president of the granting agency DFG (Deutsche Forschungsgemeinschaft) to say that he would not breathe easily until the plan was given the necessary approval by the *Länder* (which will provide 40 per cent of the funds) and written into the 1991 West German budget. A decision is expected later this year.

The programme will provide the single largest boost to West German university research since a group of new universities were built in the 1960s. DFG can expect a 12 per cent rise in 1991 and benefits will flow to the Max Planck Gesellschaft, the Alexander von Humboldt Foundation and other foundations, as well as to universities and polytechnics themselves.

By providing extra support to female scientists, Möllemann hopes to redress the huge imbalance in virtually all university faculties. Although women receive *Abitur*, the German version of a high-school diploma, in roughly equal proportion to men, just 2.6 per cent of all ('C4') professors at West German universities are women. The Education Ministry does not plan to introduce quotas for women at universities, but rather "to eliminate financial barriers" that prevent women from studying, said ministry official Wolfgang Mönikes, for instance by paying for such provisions as day care for young children.

Graduate students are another major beneficiary of the programme. The

number of graduate colleges will dramatically increase, where students will be able to undertake interdisciplinary doctoral projects supported by groups of professors and other students. The colleges will complement but not replace the traditional individual research programmes supervised by a single academic adviser or "doctor-father". Postdoctoral researchers will also do well; those who are considered excellent but cannot find a professorship will be persuaded to stay at universities through parallel programmes known as

WHERE THE NEW MONEY WILL GO

	Amount in DM
Graduate student support	>1,000 million
Postdoctoral support at non-university research centres	300 million
'Heisenberg programme' for highly qualified young researchers	175 million
Qualification for professorships (<i>Habilitation</i>)	1,000 million
Special measures for women	250 million
'Fiebigler professorships' (time-limited)	>300 million
Sending young researchers abroad (under auspices of Humboldt Foundation)	37.5 million
Strengthening European cooperation at universities	600 million
Applied research at polytechnics	200 million
Approximate total over ten years	4,000 million

'Fiebigler Professorships' and 'Heisenberg scholarships'. The first provides temporary faculty positions, including teaching responsibilities, and the second provides equipment and literature as well as a salary for researchers who will not have to teach.

Steven Dickman

MAX PLANCK GESELLSCHAFT

New problems, old worries

Lübeck, West Germany

THE Max Planck Gesellschaft (MPG) of West Germany approved the founding of two new institutes at its annual meeting here last week. Outgoing MPG president Heinz Staab announced the creation of an Institute for Microbial Ecology in Bremen and an Institute for Terrestrial Microbiology in Marburg. Construction will be completed sometime after 1992.

The two new institutes are going ahead despite continued concern that a five-year increase in funding for MPG could evaporate if Bonn and the *Länder* do not adjust the budget for inflation and higher personnel costs. The two sides agreed last year to give both MPG and the granting agency DFG (Deutsche Forschungsgemeinschaft) a five per cent increase in each of the next five years.

The MPG budget for 1990 is DM1,257 million (\$752 million). Staab expects tough negotiations with trade unions next year that could eat up the entire increase.

Freedom for Fang Lizhi

Washington

As *Nature* goes to press, Fang Lizhi, the dissident Chinese astrophysicist, and his wife, are reported to be about to land at London's Heathrow airport after having been unexpectedly granted permission to leave China. According to a statement issued by the US government, Fang is to take up a position at Cambridge University in the United Kingdom.

Fang has been living in the US embassy in Beijing since June 1989, when he sought refuge there with his wife at the time of the Tiananmen Square massacre. According to a statement released by Xinhua, the official Chinese news agency, "former Beijing Astronomical Observatory researcher Fang Lizhi" and his wife, Li Shuxian, have been given "lenient treatment" for their involvement in disturbances in China and allowed "to go abroad for medical treatment". The statement says that Fang had shown "signs of repentance".

Sidney Jones, of the human rights group Asia Watch says that permission for Fang and his wife to leave China probably came as a "reward" for US President George Bush's controversial decision to renew China's 'most-favored nation' trading status and to forestall Congressional action to remove that status. Jones hoped that Fang's release would not end international concern over the plight of other dissidents in China and that Fang would be able to speak up on their behalf.

According to Xinhua, Fang and his wife agreed that they will "not engage in political activities directed against China" after they leave the country.

Alun Anderson

Inflation is also a worry because of the costs that might arise from the unification of Germany. MPG is particularly concerned about the costs of unifying German science. Although MPG is not planning to take over any East German scientific institutions, Staab says that it intends to support large numbers of young researchers, most of whom will be expected to stay in their home institutes.

Staab also announced a programme that would encourage outstanding young researchers to remain at MPG institutes as independent researchers for a few years beyond their postdoctoral fellowships, lending flexibility to a structure that some researchers have worried was growing stiff (see *Nature* 340, 335; 1989). The meeting was the last for Staab, 64, as president. Hans Zacher, a specialist in social security and social welfare law and director of a MPG institute in Munich, began his six-year term as president on 22 June, his sixty-second birthday.

Steven Dickman

Who holds purse strings?

London

MINISTERS from countries that have ratified the 1987 Montreal Protocol are expected to agree this week to substantial reductions in the future use of chlorofluorocarbons (CFCs) and other ozone-depleting chemicals, at a London meeting to tighten the protocol. But convincing India, China and other developing countries to ratify the new protocol will be difficult if disagreements over finance to help these countries phase out CFCs are not resolved.

The United States decided earlier this month to support an international fund to help finance alternatives to CFCs in developing countries, reversing previous policy (see *Nature* 345, 193; 17 May 1990). UK Environment Secretary Chris Patten said that US action had removed a major obstacle to the success of the London meeting. But as government officials met last week to prepare an amended protocol for ministers to sign, it emerged that conditions on US participation in the fund were unacceptable to India and China.

If India and China both join the protocol, \$240 million may be needed before 1993. The fund is expected to be administered by the World Bank with advice from the United Nations Environment and Development Programme and policy determined by an executive body of 14 members, divided equally between developed and developing countries.

US delegates explained that the fund could not be an "open conduit", through which US money passes to developing countries. To ensure control over spending, the US should have a permanent seat on the executive body, and voting power should be weighted according to contributions to the fund, the delegates said. Assuming developed countries pay into the fund in proportion to their UN subscriptions, the United States will provide 25 per cent of the total.

Wang Yangzhu, who led the Chinese delegation to the preparatory meeting, said that China could not agree to the US conditions. "Every country must be equal" he said. An independent study has estimated that China will require \$42.1 million before 1993 to develop alternatives to CFCs.

Mahesh Prasad, head of the Indian delegation, also rejected the US position, and added that contributions to the fund should be linked to the (1986) figures on CFC emissions, in line with the 'polluter pays' principle. This would increase the US contribution to over 30 per cent of the total. Prasad also said that India was concerned that the establishment of an interim fund until 1993 might not be linked to a longer-term commitment. Developing countries are worried that they may be

"lured" into ratifying the protocol and then find there is "no money after three years", he said.

The negotiations were closed to the press, but an authoritative source expected the United States to back down on weighted voting in return for the assurance that decisions of the executive body must be reached by a large majority. China is expected to accept this solution, but India's position is less clear.

In comparison with the wrangles over financial management, there is broader agreement on the revised limits on future use of ozone-depleting chemicals suggested by Mostafa Tolba, executive director of the United Nations Environment Programme, with some countries pushing for even tighter limits. The Montreal Protocol currently demands that ratifying nations cut their CFC consumption by half before mid-1998, and freeze halon use at 1986 levels by 1992. Tolba's proposals call for CFCs and halons to be phased out by 2000, and adds extra restrictions intended to phase out the use of carbon tetrachloride and halve the use of methyl chloroform by the end of the century. An annex to the protocol may also be drawn up, recommending that that hydrochlorofluorocarbons (HCFCs) are not used after 2040, at the latest. HCFCs will have an important role as transitional alternatives to CFCs, but also damage the ozone layer, albeit to a much smaller extent.

PARIS MEETING

Global environment needs assessed

Paris

HEADS of space agencies from the leading industrialized nations, as well as China, met Earth scientists and politicians in Paris last week to draw up plans for future cooperation over global environment monitoring. The meeting was proposed by France at last July's 'G7' summit in Paris, and the list of recommendations will be tabled in Houston next month when the G7 nations meet again.

The meeting was intended to identify issues that will enable planned and existing Earth observation programmes to provide the data that scientists will need in order to test international models of global environmental change. As Jacques-Louis Lions, of the French national space research centre (CNES) pointed out, the success of global monitoring depends on the continuity, precision and availability of space data. Priority will need to be given to the calibration of space instruments and questions of copyright and reciprocal exchange of data must be solved. In particular, developing countries must not be excluded.

Leonard Fisk, NASA associate administrator, calculated that the international

The extent of the ozone problem was revealed in a report from the UK government-sponsored Stratospheric Ozone Review Group, chaired by John Pyle from the University of Cambridge, published last week and circulated to delegates. Speaking at the opening of the London meeting, Patten said the report made "stark reading", documenting a long-term decline in winter ozone in the Northern Hemisphere and the potential for an Arctic ozone hole.

Peter Aldhous

■ Anticipating a US phase out of HCFCs by 2015, as suggested by proposals from the US Congress, DuPont announced last week that it has frozen plans for future HCFC production. Tony Vogelsberg, environmental manager of DuPont's Freon Products Division, in London for the Montreal Protocol conference, said that chemical companies cannot justify investment in HCFC plants unless allowed to produce HCFCs until about 2030.

Vogelsberg added that the Congress proposals could be "an environmental disaster": with production of HCFCs frozen in the US, developing countries may wait until newer alternatives become available, before moving from use of CFCs, which are many times more damaging to the ozone layer than HCFCs.

Dupont, the world's leading producer of CFCs, expects to have spent \$240 million restructuring its CFC business by the end of this year, part of a \$1000 million programme. Dupont now plans to switch production to hydrofluorocarbons (HFCs), which do not deplete stratospheric ozone.

space programme for global environment monitoring will cost \$30,000 million over the next ten years. But, said Fisk, this is only about 0.01 per cent of total gross national products over the same period. NASA will provide an estimated \$17,000 million of this total, he said — about 10 per cent of the total amount the US government will spend on space exploration until 2000. Questioned about the realism of this commitment, given uncertainties in the recent past over Congress support for NASA, Fisk was confident. NASA now boasts one of the highest annual growth rates — about 15 per cent — of any of the US government agencies. "Given our present ignorance of the world and what we are doing to it, this is an effort we must make", said Fisk.

Meanwhile France, the most enthusiastic member of the European Space Agency (ESA), and keen to be seen as committed to environmental protection, has just reinforced Europe's presence in the 'Mission to Planet Earth' venture by promising to finance 23 per cent of the costs of ERS-2, ESA's next generation remote-sensing satellite.

Peter Coles

Japanese claims exaggerated

Tokyo

THE Japanese government is spending considerably less on academic research than its own figures would suggest, according to book just published by a team of British researchers. *Investing in the future: an international comparison of government funding of academic and related research* by John Irvine and co-workers at the Science Policy Research Unit of the University of Sussex concludes that figures provided by the Statistics Bureau of Japan's Management and Coordination Agency to the Organization for Economic Cooperation and Development (OECD) grossly overestimate the level of government spends on academic research. The book will add to growing Western criticism of Japan's apparent failure to contribute to basic research.

Irvine said there are major overestimates in the OECD category of General University Funds. This is the most important category for the Ministry of Education, Science and Culture (MESC), which is responsible for nearly all academic research in Japan. The Statistics Bureau gives a General University Funds total of ¥875,000 million (\$5,830 million) for 1987 but Irvine's team estimates that if a fair comparison is to be made with other countries, the figure should be only ¥535,000 million.

The discrepancy arises because the bureau includes salaries for all university staff in private, public and national universities, regardless of whether they do any research. Irvine's team suggests that only half of the salaries should be included, so that the true figure is ¥220,000 million less.

Another discrepancy stems from the inclusion by the bureau of figures for a considerable amount of "self-financed research and development" by junior colleges and technical colleges. These institutions are not included in OECD figures for other nations.

Using their corrected figures, Irvine shows that General University Funds remained at the same level in real terms between 1982 and 1986, and rose only slightly in 1987, suggesting that government claims to be spending more on basic research are exaggerated. The research funds also appear to be concentrated increasingly in areas relevant to technological development. An usually large and increasing proportion of the General University Funds (about 24 per cent) is spent on engineering. Outlays for physical sciences, which in other countries usually match or exceed that for engineering, are unusually low at 8 per cent. Irvine concludes that "domestic strategic considerations seem to have overridden the international pressures coming from the

United States and Western Europe [for Japan to increase spending on basic research]".

Researchers at Japan's National Institute of Science and Technology Policy (NISTEP) are trying to provide their own adjusted OECD figures for government and private sector spending on research and development. They agree that the figure for salary outlays for university researchers is too high, but they estimate the correction factor at 0.6 rather than the 0.5 used by Irvine's group. The NISTEP estimate may be too high, however. According to Fujio Niwa, a researcher at the institute, it is based largely on an analysis of staff at national universities who tend to carry out more research than their colleagues in private universities.

MESC officials refuse to comment on Irvine's book because they say the OECD statistics are the responsibility of the Management and Coordination Agency. But they and the ministry's academic advisers say that although MESC's overall spending on research has risen little in recent years, there have been fundamental changes in the way money is spent. In particular, the budget for competitive grants has increased significantly over the past decade. But Irvine's figures show that the budget for competitive grants is still small, only 6.2 per cent of MESC's 1989 science and technology budget, and the policy of increasing funds for these grants and an (unstated) policy of pumping more money into the new Inter-University Research Institutes, such as the High Energy Physics Laboratory in Tsukuba, has meant that university researchers have been starved of operating funds.

Academic research is not the only area where Japan may be painting an unrealistic picture of government spending. Niwa points out that government figures for outlays on energy research are "unusually large" at about 24 per cent of all government spending on research and development and are "probably too high". He says the figures include special account funds that are not entirely directed towards research. For example, a large proportion of funds from the 'Promotion of Electric Power Resources Development Special Account' are channelled to local governments to persuade local communities to accept nuclear power plants. Niwa is not yet prepared to place a figure on the overestimate. But the special accounts totalled about ¥220,000 million in 1987 and 1988, and if a large proportion of them do not qualify as research funds, this factor, combined with Irvine's corrections, will produce a significant downward revision of estimates of Japanese government spending on science.

David Swinbanks

Envious of ERATO

Tokyo

JAPAN'S Research and Development Corporation (JRDC) is pouring more money into its novel ERATO (Exploratory Research for Advanced Technology) programme, which won high praise in an assessment by foreign scientists last year (*Nature* 337, 196; 1989). Last week, JRDC announced four new projects for 1990, one more than in recent years. But as the budget for ERATO expands, Japanese scientists are beginning to question the methods by which JRDC selects recipients.

Each ERATO project provides a team of 15 to 20 young researchers (all under 35 except for the project leader) drawn from industry and academic institutions with what, in Japanese terms, is a huge grant — 1,500 to ¥2,000 million (\$10 to \$13 million) for five years.

The four newest projects are on semiconductor melts, interactions between proteins, factors underlying appetite, and mechanisms of molecular recognition. With these projects and others already under way, ERATO's budget for 1990 rises to ¥5,100 million (\$34 million), 12 per cent up on last year and about twice the level of the early to mid-1980s when ERATO's budget rose only a few per cent each year.

But university researchers are beginning to cast envious glances at the ERATO projects. Even the biggest grants from the Ministry of Education, Science, and Culture (MESC), which supports most university research, pale in size in comparison to ERATO.

But what irks some university researchers even more is the way in which recipients are selected. Unlike MESC grants, which any full-time university researcher can apply for, ERATO project leaders are selected and approached by JRDC.

JRDC officials say they select project leaders by attending symposia and conferences around Japan, by listening to what researchers have to say about people working in new areas, and by scanning the literature to see who is publishing in top international journals. The corporation then asks "several tens" of eminent university researchers to help them whittle down the short list.

Such a selection procedure, based on personal contacts, is popular in Japan because it avoids both the problem of having to choose between competing applications and the embarrassment of having to turn people down. But as the more open, competitive systems of grant selection in Western countries begin to penetrate Japan, pressure for adoption of the Western system is likely to increase.

David Swinbanks

Has database passed go?

Washington

IN the first case of its kind, Dialog Information Service, a major online information service, has sued the American Chemical Society (ACS), claiming that the society's Chemical Abstracts Service (CAS) has created an unfair monopoly for its chemistry database. The \$50 million suit charges that ACS has used "anticompetitive, illegal, and predatory acts" to control online access to CAS, which maintains the world's largest collection of scientific databases. Under US antitrust law, Dialog could be awarded triple damages, to \$150 million, if it wins its case.

The dispute revolves around two issues. The first is that, because ACS was partially subsidized by the federal government during the development of its databases, it is required to make the information in the databases freely available at a fair price, according to Dialog. ACS created much of the database software with approximately \$25 million in National Science Foundation (NSF) grants between 1965 and 1975. Since 1980, when ACS started its own online service, Dialog claims, the society has taken over more than 60 per cent of the domestic market for online access to the CAS databases, while raising its fees and royalties. ACS has also cut Dialog off from certain CAS database features, such as graphical structure searching and chemical ring data, the online service says.

The second and more significant claim is that ACS, a not-for-profit entity, has essentially become a competitor that does not pay taxes. "A not-for-profit society has a duty to benefit society and not to line its own pockets", says Robert Simmons, general counsel for Dialog. On this point, Dialog seems to be in agreement with Columbus, Ohio, the city in which CAS is located.

Columbus recently filed a complaint with the state tax commissioner to challenge ACS's real-estate tax exemption. The commission ruled in ACS's favour, but that ruling is now under appeal. CAS had total revenues last year of about \$45 million.

In support of its claim, Dialog has released excerpts from the NSF contract with ACS. The contract states that ACS "shall make publicly available at fair and reasonable prices all programs, computer-accessible files or documents . . . whether or not such programs, files or documentation shall have been developed under this contract". In response, Edward Donell, a senior CAS adviser, says "We do not think that the database is in any way a government-subsidized entity. The major cost of the database is the intellectual effort, which consumes the efforts of 450 chemists on staff." ACS nevertheless considers itself bound to the NSF contract.

"But we do not consider that making [the data] publicly available means making it available to a profit-making entity. We think we have the right to make it available to Dialog or not at our discretion", says Donell.

Dialog has compiled congressional testimony by NSF officials that appears to show clearly that NSF intended the information in NSF-sponsored databases to be available to commercial online

ORPHAN DRUG ACT

Winners to keep all

Washington

A LONG battle in the war over the revision of the Orphan Drug Act ended last week without total victory for any of the different industry groups, consumer lobbyists and members of Congress that have been fighting one another. Although Congress has yet to vote, an amendment, passed by a congressional health subcommittee would prevent companies from obtaining new market monopolies for potentially profitable orphan drugs, but would keep monopolies available for orphan drugs now on sale or under development. That means that companies such as Genentech and Amgen will continue to exercise their controversial — and lucrative — monopolies over several genetically engineered drugs already on sale.

SEISMOLOGY

Iranian earthquake

Washington

LAST week's earthquake in northern Iran, which killed as many as 40,000 people and left half a million homeless, occurred near the Caspian Sea in a complex fault zone



between the Eurasian and Iranian tectonic plates. The latest position for the epicentre is given as 37.977° N and 49.229° E with a magnitude estimated by the US Geological Survey at 7.7, is the most powerful on record for this fault zone. David Lindley

services as well as to the general public. "The language of the NSF contract makes it clear that the benefits are to be very broad", says Simmons.

Other databases and online services say they are watching the case closely. "This is only instance I can recall where an online service has sued one of its database suppliers. It says to everyone else that you've got to be careful with your clients", says Arthur Elias, director of marketing and distribution for Biosis, a company that has both a biological database and an online service. **G. Christopher Anderson**

The Orphan Drug Act was passed in 1983 to give companies incentives in the form of grants, tax credits and a seven-year marketing monopoly for the development of drugs for rare diseases — those that affect fewer than 200,000 people.

A few companies, among them Genentech and Amgen, have come under fire for allegedly charging high prices for orphan drugs such as human growth hormone (hGH) and erythropoietin.

These developments prompted Representative Henry Waxman (Democrat, California), originator of the act, to propose an amendment that would strip a company of its marketing monopoly in circumstances where another company could show it had simultaneously developed an equivalent drug. Waxman called the new arrangement 'shared exclusivity' and also proposed that orphan drug status be withdrawn altogether from drugs with a patient population greater than 200,000.

In its final version, approved last week, the amendment did contain a 'shared exclusivity' clause, but one that exempted orphan drugs that either have been granted FDA approval or those in clinical trials by 15 August 1990. The exemption covers just those drugs whose high prices had led to the amendment being proposed in the first place.

Richard Godown, president of the Industrial Biotechnology Association trade group, which opposed changes to the Orphan Drug Act, says that "shared exclusivity, no matter how it is defined, is inevitably going to deter orphan drug development in the private sector".

Supporters of the amendment saw the key issues as having been side-stepped. Abbey Myers, executive director of the National Organization for Rare Disorders, says she is pleased about the avoidance of future abuses, but warns that "if, in the future, any congressman complains to us [NORD] about the exorbitant profits that some of the orphan drug manufacturers are making, we will be able to say to them, you had your chance to fix it".

Diane Gershon

Manned mission to Mars

SIR—The cost of a manned mission to Mars is currently estimated at \$500,000 million and rising. To this must probably be added the loss of one or more flight crews, a toll that is highly likely in view of the experiences of the Apollo and shuttle programmes. So terribly dear a project requires justification commensurate with its cost, but no such justification has ever been offered, as none exists. There are no scientific grounds for the mission, it being generally agreed that robots can perform scientific tasks in space as well as, or better than, human beings, at far less cost and with no risk to life. In our time, unlike previous centuries, men do not have to be sent to explore the unknown; they can explore through the robots they create and control. A manned mission to Mars is a fifteenth century response to a twenty-first century problem.

The justifying arguments usually given for a manned mission are political and metaphysical. They include appeals to national pride, assurances that a manned Mars mission will fulfil human destiny and that collaboration with the Soviets in this venture is important for world peace, and other highly dubious claims. Bruce Murray, in his recent 'manifesto' (*Nature* **345**, 199; 1990) adds vicarious adventure to this list. He thus confirms what some of us have long believed — that public entertainment is one of the real motives for manned spaceflight.

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SIR—The US president has declared for Mars. The vice-president, head of the National Space Council, has declared, "We have seen pictures [of Mars] where there are canals, we believe, and water. If there is water, that means there is oxygen. If oxygen, that means we can breathe". Sadly, US space policy seems more appropriate for the plot of a B-movie or a 1930s pulp novel than for the energies of a world power. That policy seems to have been developed by Americans unaware of the lessons learned from the exploration of their own continent more than 400 years ago. And that policy ignores a far better goal for off-planet human presence.

Bruce Murray's article¹ advocating US-Soviet cooperation on a jaunt to Mars was not misguided. If we are to go, he reasons correctly, we should go together and share the costs. The problem is in the going. While famine, toxic waste and global climatic change are becoming accepted realities here on Earth, it makes little sense to spend the enormous human energy required to throw a handful of human beings out of one massive gravity

well only to send them plummeting down into another, onto the surface of a world so bleak that even the sixteenth century British explorer Martin Frobisher would have thought it a frigid and barren hell.

We might learn something from Frobisher's three fruitless expeditions to the New World. From 1576 to 1578, with both Crown and private backing, Frobisher led hundreds of men to the icy coasts of what is now far-northern Canada². His ships dodged ice floes, his men toiled through July and August snow storms, to bring back 1,350 tons of worthless black rocks, mistakenly thought to contain gold. Those expeditions were such a failure that they may have frightened many British investors away from financing further New World expeditions.

Now President Bush wants to send a few astronauts to Mars so that they can walk around for a bit and come back loaded with rocks. Yet modern North American space policy ignores a far more justifiable goal for human presence in space: trying to make that presence both materially and economically self-sufficient. A trip to Mars would require only an elaboration of existing technologies and an enormous infusion of government money. But a small independent colony, placed wherever it could function best, employing as yet undeveloped biotechnology to recycle wastes and grow its own food, would be an incomparable scientific achievement.

We don't need more rocks here on Earth. We need to find solutions to Earth's environmental problems. Lessons in self-sufficiency, learned by biologists and engineers in a small test-tube colony in space, might provide some of those solutions.

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2. Morison, S.E. *The European Discovery of America: The Northern Voyages* (Oxford University Press, New York, 1971).

Czech science

SIR—I wish to add to your account of Czechoslovak science (*Nature* **344**, 607–609; 1990) two further relevant points.

First, modern science has not only "marvellously . . . survived forty-two years of the old regime" as you say; it developed during Communist rule and is correspondingly biased. Institutes of the Academy of Sciences dealing with basic research (with more than 15,000 employees, in a country with 15 million inhabitants) are almost totally divorced from the universities; scientific productivity is lower than in advanced Western countries; the isolation of the country has been

disastrous for science and its economic problems made large-scale science by high-technology instruments impossible; the old regime was able to recruit scientists from the level of institutes up to the presidium of the academy who readily adapted to its requirements and managed — or mismanaged — science on behalf of the Party and Government without taking the personal risk of trying to improve the situation.

Second, not enough is said in your articles about the future. The most important needs now are: (1) to maintain, develop and optimize our actual research base in spite of continued economic and social problem; (2) to increase standards of research and productivity by increasing motivation to reach an international level, by emphasizing democratic principles in structure, management and financing, by sending creative young people (who it is hoped would return) to the best laboratories abroad and by removing all obstacles (including the financial ones) to scientific communication and travelling; and (3) to provide from elsewhere, qualified help (mostly to its badly retarded technology, education, health care and ecology) as well as to contribute to a general improvement of cultural, moral and humanitarian standards.

Although all this requires mainly that we work more and better, solidarity and support from the international scientific community would be of great value. In addition, research grants making possible the acquisition of modern instrumentation, travel, postgraduate training, attendance at conferences and so on, and the publication of good scientific papers by international journals would greatly contribute to European and worldwide integration of Czechoslovak science.

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European projects

SIR—In your leading article "Metrication and the falling yen" (*Nature* **344**, 575; 1990), you refer to "the Hermes spacecraft" that "will be built in France".

Hermes is a programme of the European Space Agency and the correct statement is that it is built mainly in Europe. My comment is not intended to diminish the role of France but I should like to remind you of the European nature of programmes such as Hermes, Ariane, Airbus and many more.

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One-dimensional solids made tractable

Schemes for using organic polymers as electrical conductors have been much dreamed of, but recent calculations suggest there is some way to go before one-dimensional solids are as simple as they seem.

POLYACETYLENE might be thought the simplest of polymers, consisting as it does simply of a chain of carbon atoms, each carrying a single hydrogen atom. And the simplest form of polyacetylene is that called *trans*, in which the successive carbon-carbon bonds zig-zag regularly to form a structure which necessarily (in the absence of stress) lies in a plane, as do the hydrogen bonds to each carbon, which lie alternately on one side of the skeleton and the other. But, of course, things are never as simple as they seem.

First, there is the little matter of the spacings between successive carbon atoms, which are joined by alternating single and double bonds, as are the atoms in a benzene ring. The expectation would be that all the bonds in such an array would be of equal length, as indeed they are in benzene and in the two-dimensional sheets of carbon atoms in graphite. In the argot of the chemistry textbooks, the four potentially bonding electrons on each carbon atom would be organized into three σ -orbitals (one towards each of two carbon neighbours and one towards a hydrogen) with the fourth occupying a familiar π -orbital.

More than thirty years have passed since Rudolf Peierls argued that *trans*-polyacetylene (like other linear conjugated structures) is not necessarily that symmetrical. There are two potentially disturbing influences, of which the chief is the way that electric charge can slop about from one place to another in such a system. Physically, the explanation is that the energetic penalty in removing one π -electron from a single site is a fixed amount, but the effect is to change the bond configuration in one or other of the remaining halves of the whole molecule.

Alternatively, the energetic penalty in creating charge separations by as much as the whole length of the molecule is fixed, and (for long molecules) small compared with the statistical benefit of the accessibility of a whole new range of states. Yet another way of putting this point is to recognize that the electrons in such a structure form electronic bands which are in this case half-filled (so that *trans*-polyacetylene molecules are electrically conducting), when charge separation amounts to the promotion of one electron into the conducting zone and the creation of a corresponding electron hole.

Peierls dealt with the disturbance of symmetry caused by the flopping about of electric charge with the help of what he

called charge-density waves. Their effect is to break the expected symmetry of polyacetylene, meaning that the length of carbon-carbon bonds oscillates from one site to the next. More strictly, there is a phase transition, appropriately called the Peierls transition, from the symmetrical state to that of the poly-dimer. The short and long bonds in this alternating pattern are respectively 1.36 Å and 1.46 Å in length.

Ambitions to design organic conductors with predetermined properties are everywhere. The hope that there will one day be organic superconductors has not everywhere vanished. Polyacetylene is not so much a prototype of these materials, but a test-bed. It has the convenient property that it can be systematically doped, as semiconductors are, most conveniently by substituting electro-positive or electro-negative groups for hydrogen atoms along the length of a polymer. Electron donors will add (negative) electric charge to the conduction bands, electron acceptors will subtract it.

The remarkable truth, made plain by measurement, is that the properties of polyacetylene are remarkably sensitive to the degree of charge transfer, or of doping. The average length of a repeating unit can change by several per cent by alternations of doping that imply charge transfers of 20 per cent of an electron charge from one carbon atom to its neighbour. Elastic properties, notably Young's modulus, are similarly sensitive not only to the degree of doping, but to whether the doping is by electron acceptor or donor groups.

The measurements are confusing, perhaps because there are not enough of them, but more probably because there is not a sufficiently well-developed framework of reasonable expectation. But people seem aware of what is needed. Two groups have recently reported attempts to calculate the properties of doped polyacetylene and such materials. Sung Y. Hong and Miklos Kertesz from Georgetown University (Washington, D.C.) appear to have gone a long way to account for recorded measurements of polyacetylene (*Phys. Rev. Lett.* **64**, 3031; 18 June 1990).

To calculate elastic constants, the first need is to estimate how the energy surface varies in the neighbourhood of equilibrium as a function of parameters describing the structure of the molecule. Hong and Kertesz do this in two ways, by using

an infinite molecular chain and one consisting of 21 carbon atoms (with the ends of the chain sealed off with extra hydrogen atoms). Both schemes rely on the computer codes which have become the bread and butter of computational chemistry. The outcome is satisfying in two respects — the predicted variations of Young's modulus with doping is not merely of the same order of magnitude as the measurements, but the qualitative variation of the modulus with the degree of doping is that found in practice, even to the extent that the asymmetry with the sense of the doping is reproduced.

Joseph A. Aronovitz, Paul Goldbart and George Mozurkewich from the University of Illinois at Urbana-Champaign set out to demonstrate a more subtle point by a more general argument (*Phys. Rev. Lett.* **64**, 2799; 4 June 1990). Their interest is not simple polyacetylene, but the mostly inorganic materials such as $\text{K}_{0.3}\text{MoO}_3$, which is called blue-bronze, and $(\text{TaSe}_4)_2\text{I}$, both of which form solid crystals consisting of parallel strings of atoms which can act as conductors and which support charge-density waves just as if they were polyacetylene. Both materials have abnormally high thermal conductivity as well as specific heat anomalies near their Peierls transitions, at 180 K and 263 K respectively.

What Aronovitz, Goldbart and Mozurkewich have done is to consider the interaction between lattice vibrations and charge-density waves, which are known to be coupled together strongly near the transition point. The argument follows the outlines of V. L. Ginzburg's treatment of phase transitions in superconducting solids, for example, but one conclusion is that the behaviour of these one-dimensional solids resembles, near the transition point, the phenomenon of magnetoelasticity.

The upshot of an intricate argument is that changes of the elastic constants near the Peierls transition should be related in a simple (and calculable) way to the anomaly in the specific heat, which on a graph of specific heat against temperature lies like a cusp above a smooth background (which shows that the entropy at the transition is finite). The further remarkable prediction is that there should be similar anomalies of the elastic constants near the transitions, which have yet to be observed. If the prediction is confirmed, who will be the first to make a device of it? And what for?

John Maddox

Could Arctic ice be thinning?

A. S. McLaren, R. G. Barry & R. H. Bourke

GLOBAL warming in high latitudes, the predicted result of increasing greenhouse-gas emissions, could have important consequences for Arctic sea ice. Melting of the ice would lead to a decrease in both thickness and areal extent, and to more openings within the ice sheets. Most of the recent data on sea-ice thinning and opening have been provided by sonar measurements taken under the ice by submarines; they have not yet revealed any conclusive evidence of a trend towards widespread thinning. On page 795 of this issue¹, Wadhams presents data collected north of Greenland by British submarines in October 1976 and May 1987, and shows that the mean sea-ice thickness is significantly less in the later year. The observation is provocative, but more data are required before one can hope to discern overall trends with any confidence.

The complex mixture of open water and ice of varying type and thickness that makes up the Arctic Ocean is a significant component of the global climate system. The geographical extent and thickness of the sea ice control the amounts of heat, moisture and momentum exchanged between the Arctic Ocean and the atmosphere. Climate models² suggest that a doubling in levels of atmospheric CO₂ should produce significant changes in the distribution of polar sea-ice extent and thickness. In turn, such changes would affect atmosphere-sea interactions and would have important implications for the climate of the Northern Hemisphere.

Since the late 1800s, when the earliest measurements of Arctic sea-ice thickness were taken, estimates of the overall mean thickness of pack ice have hovered around 3.0 m. Nansen³ published the first measurements of Arctic basin sea-ice thicknesses collected during his voyage of 1893–1896 in the *Fram*. He reported an average thickness of floes in the region of the Transpolar Drift ranging from 3.1 to 3.8 m. Field measurements^{4,5} by groups in the Soviet Union indicated that old pack ice was not less than 3.0 m thick, that perennial ice ranged between 2.3 and 3.3 m, and that the maximum thickness of non-ridged sea ice was between 3.0 and 5.0 m. Thickness measurements taken during the 1968/69 British Trans-Arctic Expedition⁶ gave an overall average of 4.2 m; later papers⁷ provided revised estimates of 4.4–4.6 m and 3.7–3.9 m from the Pacific and Eurasian sides of the Arctic respectively, and a mean overall pack-ice thickness across the Arctic Basin of 3.7 m (ref. 8). But these field measurements, collected by drilling several hundred holes through the ice, provide an uncertain representation of the Arctic Ocean ice

cover, which in places is heavily ridged and hummocked through wind-forced convergence and rafting of floes.

Since 1958⁹, it has been possible to measure thicknesses using submarine sonar beneath the ice. The size of the 'footprint' sampled by the under-ice sonar depends on the instrument frequency/beam-width ratio and the operating depth of the submarine. These observations are calibrated for the local sea level by measurements in areas of open water (leads and polynyas).

Based on US submarine cruise results for 1960 and 1962, Wittmann and Schule¹⁰ reported that the average Arctic pack-ice thickness was between 2.4 and 4.7 m with an overall average of 3.9 m. Swithinbank¹¹, Williams *et al.*¹², Wadhams¹³ and McLaren^{9,14} reported sea-ice thickness distributions from four Arctic basin-wide submarine cruises, including those of the USS *Nautilus* (1958), USS *Queenfish* (1970), HMS *Dreadnought* (1971) and HMS *Sovereign* (1976). But none of these reports addressed the issue of trends in ice thickness. As each of the voyages travelled along different routes in different seasons and through various geographical areas, and in addition as there were differences in equipment and measurement accuracies, it was not possible to establish a reliable baseline against which to identify any trend.

There are few published data on the variability of Arctic ice thickness. Bourke and Garrett¹⁵ have presented seasonal climatologies of ice thickness in the Arctic Basin, deduced from unclassified submarine sonar data, but they made no reference to variability. McLaren¹¹ compared ice thickness along the same basin-wide transect during early August in two different years (1958 and 1970) by using submarine sonar data from different cruises. He found that ice was thinner across 941 km of the Canada Basin in 1970 (mean thickness of 2.39 m) relative to 1958 (mean of 3.08 m). Although the data from the two cruises varied considerably in overall quality, they were of similar quality across the Canada Basin, making this 0.69-m difference significant.

Ice in the Canada Basin tends to circulate in a clockwise sense, but during the late summer this motion is reversed owing to the development of atmospheric cyclones^{16,17}. Such changes in ice motion lead to opening up of the ice, lowering concentrations by 20–30 per cent¹⁸ relative to the previous season. If a reversal of this sort occurred in 1970 but not in 1958, the thinning observed by McLaren would be difficult to explain in terms of normal synoptic and interannual variability.

Unfortunately, the available atmospheric-pressure data for those years lack the necessary accuracy to determine this.

Wadhams' observation of thinning of the Arctic ice cover north of Greenland in 1987 relative to 1976 raises similar questions: is the Arctic ice really beginning to thin, or are there still insufficient available data to rule out normal synoptic and interannual variability? Recent studies¹⁹ of sea-ice extent in the Arctic basin, based on satellite passive microwave data, have attempted to determine changes in ice cover that might indicate warming or cooling trends. Variations in global sea-ice extent remotely sensed by the Nimbus 7 satellite during 1978–1987 indicate no significant trends²⁰; neither do data for 1973–1987 for sea-ice extent in the Northern Hemisphere²¹.

The fact is that no data published so far can provide a reliable indication of whether the sea ice of the Arctic Basin is thinning, thickening or remaining essentially constant. Wadhams rightly stresses the importance of monitoring Arctic ice thickness on a systematic basis. A direct approach would involve statistical analysis by season, region and character of atmospheric circulation for each year of all the high-quality, comparable, basin-wide under-ice thickness distribution data obtained by US and British nuclear submarines since 1958. Only then might genuine trends be distinguished from natural variability. □

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BOVINE SPONGIFORM ENCEPHALOPATHY

Taking stock of the issues

Richard H. Kimberlin

THE controversy over bovine spongiform encephalopathy (BSE) remains the subject of intense public interest in Britain. Popularly known as 'mad cow disease', BSE afflicts cattle and was first identified in November 1986¹. Last month, media coverage of the disease exploded. There was the report of a Siamese cat from the Bristol area with a spongiform encephalopathy like that of the bovine form and that caused by scrapie; two more cases have since been confirmed. Some local authorities took beef off school menus. And France, Germany and Italy banned the import of British beef, a dispute that, at the time of going to press, had been resolved.

There is no doubt that BSE is a new member of the scrapie family of diseases. Like scrapie, it affects the brain, producing the same type of vacuolar (spongiform) lesions¹ and also disease-specific fibrils which are derived from a normal glycoprotein called PrP (ref. 2). BSE has been transmitted by injection to both cattle³ and mice⁴, after long incubation periods. Transmission to mice⁵ has also been achieved by feeding them large amounts of infected brain and the resulting disease is very like murine scrapie. It is important that these similarities began to emerge as soon as BSE was first recognized, because it meant that scientists were on familiar ground.

Indeed, the occurrence of BSE was not without precedent. In 1965, a disease called transmissible mink encephalopathy (TME) was identified⁶. This rare disease is caused by an exogenous source of infection to which ranch-reared mink become exposed through contaminated feed (which, not uncommonly, includes unprocessed offals and dead stock). Sheep and goats are the only known natural reservoirs of scrapie-like agents in animals⁷ and therefore a causal link between scrapie and TME is highly likely even though it was not possible to prove the feeding of sheep material in some outbreaks.

The outbreak of BSE started in a similar way, but with two major differences. First, the source of infection was not unprocessed offals but materials that had been converted into meat and bone meal, and used as a protein-rich feed supplement⁸. Second, exposure to the infectious agent was not local and episodic, but widespread and continuous, running from around 1981–82 until July 1988 when the British government banned the feeding of meat and bone meal to all ruminants.

The early stages of the epidemic gave the impression of an uncontrollable

'spread' of the disease from the south of England to the rest of Britain. But in retrospect it is clear that exposure through feed, sufficient to cause the disease in cattle, started more or less simultaneously in many parts of the country, though with a higher exposure in the south. The illusory 'spread' of the disease was due to the passage of time allowing more and more primarily infected cattle to show clinical symptoms. Even after more than 14,000 cases, 95 per cent of all



The BSE epidemic has given rise to humour as well as concern. Reproduced here is a cartoon from the *Daily Telegraph* of 18 May.

herds have not had a case, about two-thirds of all affected herds have only had one case, and there is still no evidence for cattle-to-cattle spread of infection.

The estimated incubation period of BSE ranges from about 2.5 years to over 8 years⁹. This means that cattle were being infected for at least four years before the disease was even recognized. The long incubation period also means that most of the cases occurring now acquired the infection long before the ruminant protein ban was put into effect.

Given the large sheep population in Britain, and that scrapie is endemic here, it seems that the potential for BSE to occur has existed for a long time. It just required the right combination of factors (sheep, scrapie and altered rendering practices) to increase the exposure to scrapie infection to a level high enough to cause the disease in cattle. At the same time, BSE nearly didn't occur at all; the large number of cases is a reflection of

the size of the cattle industry not the incidence of disease in the national herd, which still averages only about three cases per 1,000 adults and contrasts with the near 100 per cent morbidity among breeding adults in some outbreaks of TME⁶. Perhaps it is not surprising that BSE has occurred only in the British Isles (and in a few exported cattle), even though scrapie is endemic among sheep in several other countries where meat and bone meal is also used as a feed additive.

The BSE epidemic may take either of two courses, each of them having a precedent in the scrapie family of diseases. The first comes from TME, which spontaneously self-limits because there are no natural routes for the infection to spread from mink to mink (the exception is if cannibalism occurs)⁶. If cattle are also 'dead-end' hosts for infection, then the ban on ruminant protein is all that is necessary for the disappearance of BSE. But long incubation periods mean that eradication could take until the end of the century.

The alternative course is that BSE may turn into an endemic infection of cattle, as scrapie is of sheep. Scrapie is transmitted maternally, from ewe to lamb, and also horizontally between unrelated sheep⁷. If BSE can spread naturally within the cattle population, the course of the outbreak will depend on the efficiencies of maternal and horizontal transmission. The worst case is that high incidence of horizontal spread could make eradication of BSE as difficult as it is with scrapie.

The public health issues have naturally been the centre of attention. Here again, vital information comes from the scrapie family; the family includes at least two diseases of man, of which Creutzfeldt-Jakob disease (CJD) is the most relevant⁷.

The scrapie-like diseases are not highly infectious — the 'dead-end' nature of TME is an example — but they can be transmitted by injection to other species (including some primates), and even by feeding large amounts of infected material⁹. This does not mean that scrapie necessarily causes CJD in man, but the possibility had to be considered once the experimental transmissibility of CJD to animals was shown in 1968.

Typically, CJD is sporadic with a remarkably uniform incidence worldwide of about one per million of population per year. Its occurrence does not match the world distribution of sheep, scrapie or the consumption of sheep products, nor has any link been established between the incidence of CJD and eating habits (for example the regular consumption of brain), environment (urban or rural) or various occupations such as those of shepherds, butchers and veterinarians¹⁰. Man has undoubtedly been exposed to scrapie infection for a very

long time. So if the agent causing BSE in cattle is effectively the same as that causing scrapie in sheep, the risks of BSE to public health will be equally low.

The other possibility comes from laboratory studies showing that strain selection sometimes occurs when crossing the 'species barrier'¹¹. Scrapie infection of cattle could have led to the selection of a mutant strain. The difficulty is that there is no easy way of knowing whether any such mutant is as nonpathogenic to man as sheep scrapie, or more pathogenic.

An immediate solution to the dilemma is to exploit the understanding of the pathogenesis of these diseases. One of the reasons for their slowness has to do with the limited capacity for cell-to-cell spread of infection and the fact that most tissues do not support multiplication of the agent⁷. The risks to man would not be from milk or meat, in which infectivity is undetectable, even at the clinical stage of the natural disease¹². This is why the health authorities who have taken beef off the menu might reconsider. Whatever risk there may be would come from the largest organs in which infection can multiply the most, namely brain, spinal cord, spleen, thymus, tonsil and certain parts of the intestines. These tissues are the specified offals which are now excluded from human food as a pre-emptive measure; offals from calves are exempted from the ban because it takes several months for scrapie multiplication to get underway, and the amounts of agent in young lambs are too low to be detected.

The advent of natural cases of spongiform encephalopathy in cats has been a recognized possibility ever since cats were shown to be susceptible to high doses of agent injected into the brain. Again, it is a matter of natural exposure and the dose. At the moment, the cause of the cat disease is not known; it could be due to infection with scrapie or BSE, or even an unrecognized endemic infection of cats. But it is worth noting that most of the pet-food industry applied a voluntary ban on use of offals at about the same time as the specified offals were banned from human food.

The British government has allocated about £12 million to BSE research over the next three years. Much of this money will be spent on projects relevant to the eradication of the disease, including a key study to determine whether or not it is naturally transmissible within the cattle population. But whatever the future course of BSE, eradication would be much quicker with an efficient diagnostic test to identify infected animals long before they show clinical signs.

Two main approaches are being tried. One is based on detecting the specific fibrils by which the scrapie-like diseases can be recognized¹. These fibrils consist of a post-translationally modified form of

PrP (ref. 13), and they accumulate in large quantities in diseased brains¹⁴. But it is one thing to detect the fibrils in brain after the animal has been killed, quite another to find them (assuming they are there) in, say, blood samples from the live animal. The challenge is to develop tests that are sensitive enough to detect the much smaller amounts of modified PrP that are found outside brain, and with a specificity that can distinguish between the normal and modified forms. A big step forward would be to identify precisely the physico-chemical differences between the two¹⁵.

The other approach is a test that detects the genome of the infectious agent, which undoubtedly exists⁷ and is, by definition, specific to the agent. But the nature of the genome is a matter of continuing controversy. Views about it range from its being a very small nucleic acid which does not code for protein but forms an infectious agent by combining with a host protein such as PrP (the 'virino' hypothesis¹⁶), to the heretical notion that it is an infectious self-replicating protein (the 'prion' hypothesis¹⁷), with modified PrP as a prime candidate.

GEOMORPHOLOGY

Big rills have little rills . . .

John Thornes

If deprived of vegetation cover and human artefacts, drainage channels seem to share in common a self-similar, 'fractal' structure. A network of rills and gullies seen in aerial photographs of 'badland' terrains looks much like the Amazon basin seen on a satellite image. Consequently, badlands have been studied as surrogates for landforms as a whole¹, and have been developed as 'experimental hardware' both in the laboratory² and in the field. These studies indicate that a strong coupling exists between the evolution of the land surface and the development of the channel network. This coupling is often masked in large river basins by geological complexity, tectonic activity, and the effects of glaciation and of human activity. The coupling is complex: water

If anything good can be said to have come out of the BSE epidemic, it is that it has stimulated a great deal of research on these enigmatic agents. □

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and sediment produced by erosion create and transform the channels, while changes in the long profile of streams alter the gradients of hillslopes to either side. These effects have been revealed experimentally³ and in digital simulations⁴; now Newmann and Turcotte⁵ have provided an important step towards a quantitative theoretical model of landscape evolution which incorporates this coupling.

Drainage channels and hillslope surfaces are spatially coupled, as indicated by the pattern of elevation revealed by ground-surveyed contours or by digital terrain models obtained from remotely sensed images. Transects of elevation across a terrain can be used to obtain the fractal (Hausdorff) dimension of self-similar landforms, through techniques



Question of scale — a small gully crossing an agricultural terrace in semi-arid Spain could, without vegetation to set the scale, be mistaken for an aerial view of the Grand Canyon.

such as the varying-step-length (Richardson) method⁶ or semi-variogram analysis⁷ or through the relationship between spectral energy density and wavenumber in the size spectrum of landscape features. Newmann and Turcotte⁸ argue that transects across a variety of different terrains give Hausdorff dimensions of 1.5, implying that the landforms are indeed fractal, and furthermore that they are 'brownian', meaning that their power spectra resemble those generated by brownian motion. They show that Culling's⁸ linear-diffusion model cannot give a realistic description of landscape development over long time-scales because it predicts that, as development proceeds, small-wavelength features start to disappear; this would lead to a loss of self-similarity.

Newmann and Turcotte begin from the assumption, based on their observations of real landscapes, that the topography must have brownian properties. This can be guaranteed by requiring that the rate of decay of the mean-squared height of a feature with a particular wavelength is the product of its existing height and a rate coefficient. This means that the relationship is nonlinear. The rate coefficient is a function of successive wavenumbers and the model is therefore based on the same basic proposition as the Kolmogorov-Obukhov cascade, which is used to model the structure of energy dissipation in turbulent fluid flow. The effects of non-linearity are transmitted down the system from the largest to the smallest length scales; at equilibrium this means that the system is scale-invariant and the rate coefficients are constant. This is true even if the initial amplitudes are not self-similar — the self-similarity imposed by the two requirements itself cascades down through the system to the smallest elements.

Newmann and Turcotte provide no physical mechanism for this behaviour, but they suggest that the nonlinear, constant readjustment of mean-square heights is in some manner related to the frequency of storm events of different magnitude. This would require a means of transforming a temporally varying erosional force, dependent on the extent of storm rainfall, into a spatially self-similar landform. At present there seems to be no justification for this, although, as Kirkby⁹ has implied, the two are not unrelated.

Geomorphologists generally have viewed landform evolution as the product of rainfall and runoff, proceeding by means of channel development, the elimination of small rills through capture by larger channels, and channel joining. Horton¹⁰, for example, proposed such a model for the evolution of drainage patterns on an emerging lake floor. This might be called a 'top-down' approach, in the sense that the dominant network development is believed to take place

through random joining of flows formed by an excess of rainfall over infiltration. Recently, however, it has become evident that conditions at the head of channels play an important role in their evolution; in other words, development is 'base-level-controlled' and propagation of streams occurs by headward erosion. In Bristol we have simulated by experiment and computer the headward bifurcation and growth of gully systems. This requires a continuous lowering of channel-head topography and a continuous energy input² both from rainfall and from channel incision by basal lowering. If a given wavenumber in the Newmann and Turcotte model represents a stream of given order (a geometrical property of the network), energy and organization cascade down from the major drainage lines to progressively lower-order streams. The process stops only when there is insufficient area to sustain a stream channel. The system is self-reinforcing, because a more dense network speeds up the passage of water waves, which are correspondingly more peaked and more erosive, until finally a self-similar equilibrium is reached.

Newmann and Turcotte are taking a new direction in suggesting that the cascade is the appropriate way of describing how landscapes evolve. The initial assumption that all landscapes are self-similar is probably more contentious. Certainly, as Culling showed¹¹ before his recent untimely death, soil-covered land-

scapes dominated by creep are significantly smoother than brownian (having Hausdorff dimensions significantly smaller than 1.5), whereas regenerating channel systems are somewhat rougher. In other words, the gullied wash-process-controlled areas such as badlands and the types of terrain that provided Newmann and Turcotte with their data are particularly appropriate for the development of the cascade model. The boundaries between domains controlled by other processes should be delineated by a change in the fractal dimension of their morphology.

To paraphrase L.F. Richardson, it may be that:

*Big rills have little rills formed by bifurcation,
and little rills have smaller rills 'til spatial
saturation.*

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PALAEONTOLOGY

The solution to a jigsaw puzzle

Stefan Bengtson

AN extraordinary discovery by Conway Morris and Peel, described on page 802 of this issue¹, answers the prayers of many palaeontologists. The authors report complete specimens of halkieriids from 550-million-year-old Early Cambrian rocks in northern Greenland.

Those unfamiliar with halkieriids may be excused. The first fragment was unearthed on Bornholm in the Baltic area in the 1960s (ref. 2), and it took some time before palaeontologists realized that they were dealing with isolated dermal scales (sclerites) of a previously unknown type of animal. There are no halkieriids alive today; in their short time they were highly successful and filled the Earth's seas.

Palaeontologists are traditionally famous (or infamous) for reconstructing whole animals from the debris of death. Mostly they cheat. Even extinct beasts such as dinosaurs have scores of living relatives (birds, mammals, reptiles) that make reconstructions 'simply' a matter of competent comparative anatomy. But how do you go about the job when there seem

to be no close living relatives on which to base the model? This is a problem particularly when dealing with organisms that derive from the 'Cambrian explosion'.

If any event in life's history resembles man's creation myths, it is this sudden diversification of marine life when multicellular organisms took over as the dominant actors in ecology and evolution. Baffling (and embarrassing) to Darwin, this event still dazzles us and stands as a major biological revolution on a par with the invention of self-replication and the origin of the eukaryotic cell. The animal phyla emerged out of the Precambrian mists with most of the attributes of their modern descendants. But nature is wasteful. Most species never give rise to anything, and present-day phyla derive from a lucky minority. Many of the not-so-lucky fossil species may also be comfortably classified in these same living phyla, but it is a feature of many Cambrian assemblages that they contain a large proportion of forms that cannot be so treated. In the 1970s, the realization started to grow that these poorly understood forms may indi-

cate a great diversity of high-level taxa³⁻⁵.

To work out the biology and anatomy of these early offshoots of the phylogenetic bush is a formidable task. Most of the fossils consist either of shells comparable to those we know from molluscs, or of dissociated sclerites of composite skeletons. With nothing more than bits and pieces to work from, and no known relative to use for comparison, we often have to be content with trying to analyse which sclerites belong to which species, how the skeletal tissue was formed, and how the complete skeleton may have been constructed in order to work as an integrated unit. For a compelling answer about anatomy and affinities there are just too many unknowns. Dreams of finding the complete specimen of any one of a host of bizarre groups — tomotiids, cambroclaves, halkieriids — are the *fata Morgana* of Cambrian palaeobiologists.

And now we are given the complete halkieriid. In many ways it looks as expected⁶: a sluglike animal with a dorsal armour consisting of several types of imbricating scales. But the presence of two large shells, one in each end, comes as a complete surprise and breaks the pattern of uniformity. Where did they come from? Are they memories of an earlier bivalved condition, or have they evolved secondarily to meet a more direct need, such as protecting the entrances of a U-shaped burrow?

The shells were unexpected for several reasons: they do not have an obvious function; they are absent from the related *Wiwaxia*⁷; they do not have surfaces that interlock with other elements; and their accretionary mode of growth contrasts with the mode of formation of the sclerites. Nevertheless, they could have been predicted — and in a way they were. Missarzhevsky⁸ pointed out that certain early cap-shaped shells (known as *Mai-khanella*) appear to consist of coalesced scales. He suggested that shells of monoplacophoran molluscs evolved through the merging of scales in organisms closely related to halkieriids.

This is a near miss. To view halkieriids as early molluscs is stretching the evidence; to have them as monoplacophorans is breaking it. But a mollusc

connection seems not unlikely, as Conway Morris and Peel point out, and the idea that shells can form through the merging of sclerites is not an outlandish (or even new) one. In any case, the finding of complete halkieriids reopens investigation into the nature of various Cambrian cap-shaped shells. It raises fundamental questions about the origin of calcareous skeletons, in particular the generic relationship between isolated sclerites and shells growing through marginal accretion. And it demonstrates just how precarious reconstructions of unknown organisms may be, and suggests that for such work we must employ a combination of vivid imagination, scrupulous pedantry and deep humility.

DEVELOPMENTAL BIOLOGY

Reading the retinoid signals

Jeremy Brookes

RETINOIC acid (RA) and its immediate precursor retinoids are able to respecify positional identity on an axis in development and regeneration. This remarkable and rare effect is graded and dose-dependent, in that the positional value is altered continuously within a certain concentration range. To date there are only two developing systems in which this property has been clearly demonstrated: the antero-posterior (AP) axis in the chick limb bud, and various axes, most notably the proximo-distal (PD), in the urodele amphibian limb regeneration blastema¹.

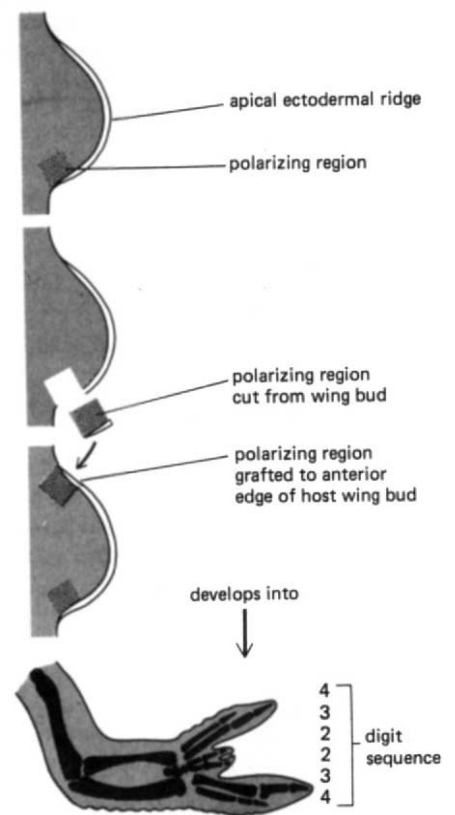
In the chick limb bud, topical application of RA to the anterior margin has many of the consequences of grafting the natural polarizing region from the posterior margin^{2,3} (see figure). The possibility that RA is an endogenous morphogen released by the polarizing region was given impetus by the identification of RA in plausible amounts in the limb bud, and by evidence that there are somewhat higher levels in posterior rather than anterior fragments⁴. Studies of the mechanism of action of RA in positional respecification should lead to insights into how position is encoded along a vertebrate axis, and research in this field gathered additional momentum with the identification of RA receptors that are ligand-dependent transcription factors of the steroid/thyroid hormone superfamily^{5,6}. Two papers on pages 815 and 819 of this issue — one by Thaller and Eichele⁷, the other by Wagner *et al.*⁸ — add to the diversity of retinoids that may be active in development and to that of systems on which they may act. Together with other recent reports, they raise intriguing questions about the status of the retinoids as endogenous morphogens.

On page 815, Thaller and Eichele⁷ demonstrate that the retinoid 3,4-dide-

A parallel case is even more sobering. The netlike phosphatic plates of *Microdictyon* were known from Cambrian microfossil samples all over the world⁹. Some attempts had been made to interpret their function, but a recent discovery¹⁰ in China of appendiculate worm-like bodies with a pair of these plates at the base of each appendage (or appendage pair) suggests something from another planet. Nature has a way of outshining our most speculative hypotheses, and we have a long way to go before we have a full view of Earth's early metazoans. □

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hydroretinoic acid (ddRA) is present in significant quantities in the chick embryo and is able to induce AP duplications in the limb bud with potency comparable to that of RA. The authors provide evidence that this activity is not dependent on meta-



A small zone known as the polarizing region on the posterior margin of the chick limb bud is able, when transplanted onto the anterior margin, to induce duplication of the pattern of digits. It is therefore believed to specify the positional values that dictate the digit pattern, possibly by releasing retinoic acid that acts as a graded morphogenetic signal. (From *Molecular Biology of the Cell*, 2nd edn)

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bolism to RA, and that synthesis of ddRA proceeds by dehydrogenation of retinol followed by sequential oxidations. ddRA is clearly a player of potential significance because its concentration in the limb bud is about six times greater than that of RA, so it will be important to determine if analysis of anterior and posterior fragments shows a difference in its level.

The chick limb bud assay has been used for some time to analyse other regions of the chick embryo for polarizing activity, and Hensen's node and the notochord are both able to evoke AP duplications⁹. Wagner *et al.*⁸ now report that the floor plate, an epithelial structure at the ventral midline of the developing spinal cord, has polarizing activity. The floor plate may play a part in neural-tube development, and in the formation of certain axonal projections¹⁰. Regions of developing neural tissue lacking the floor plate, including other regions of the spinal cord, have little or no activity, and the activity appears around the time of neural-tube closure before differentiation begins. So is it RA that is responsible?

As there is not enough tissue to analyse endogenous retinoids directly, the authors used metabolic labelling with tritiated retinol and analysed the production of RA and didehydroretinol. Both floor plate and neural tube without floor plate produce these metabolites, but at a somewhat lower specific converting activity for the neural tube. There is thus some disparity between the polarizing-region assays and the metabolic studies, and the authors suggest this may be resolved by the ability of the floor plate to accumulate retinol through the retinol-building protein. These questions remind us that the evidence that RA or ddRA are endogenous morphogens in the limb bud is not yet compelling, and is even less so for the urodele blastema. For example, more evidence is needed to confirm that there are continuous variations in retinoid levels or in cellular responsiveness¹¹, and that such variations actually determine the axial positions of cells.

The morphogenetic effects of RA on the limb produce structures that have normal morphology but that arise at an abnormal position on the developmental axis. It is not clear how widespread such effects will be, but they are distinct, at least in their consequences, from the traditional teratogenic effects of retinoic acid. A recent paper¹² on induction of cleft palate in rodents illustrates that retinoids can act on diverse cell types and influence cell interactions.

At day 8 in the mouse embryo, cranial neural crest cells migrate to form the maxillary prominence from which the palatal shelves form. Exposure to RA at this stage affects both survival and migration of crest cells. Exposure of the palatal shelves from day 10 onwards has stage-

The floods in eastern Australia

MAJOR floods are a fact of life in inland eastern Australia. Those in April represented the eighth such event since 1950. But they were also the most extensive on record, claiming five lives and causing hundreds of millions of dollars worth of damage to crops, livestock and farm equipment. The damage to some towns is so great that they may not survive in

the long term. River levels in the region rose up to 12 metres above normal, and by 24 April more than 220,000 km² of southern Queensland and northern New South Wales were submerged beneath a vast inland sea.

The picture is a satellite image of the floods of April 1990, produced from NOAA-AVHRR satellite data collected on 24 April 1990. It shows the region from the tip of Cape York through western and central Queensland to western New South Wales. Red represents active river channels; yellow indicates standing water more than 1m deep.

Inland eastern Australia lies within the catchments of three large drainage basins: the Darling to the east (largely draining the Eastern Highlands), and the Bulloo-Bancannia and Lake Eyre basins to the west (the latter fed largely by the Diamantina and Cooper Rivers).

The susceptibility of this area to widespread flooding is a consequence of the unusual hydrological characteristics of these rivers. Their behaviour differs markedly from that of humid river systems. First, they show remarkable flow variability, with up to a thousand times more variation in mean annual discharge than in most European and North American rivers. Second, the active channel systems are able to

expand to exceptional widths during

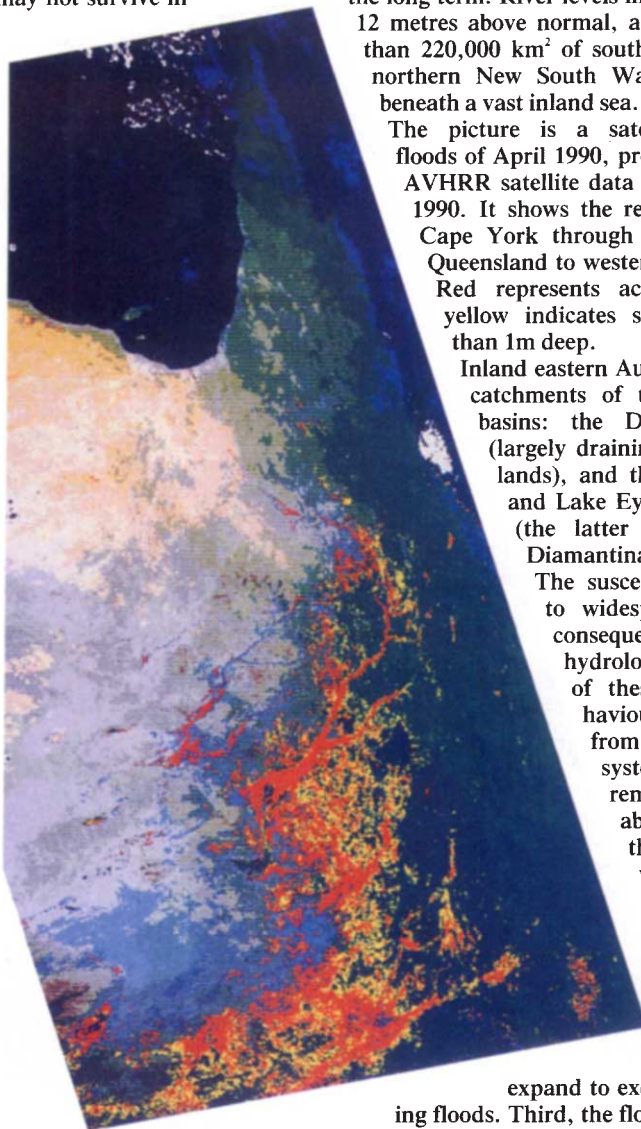
floods. Third, the flood discharge tends to decrease systematically downstream. Fourth, the flood pulses are of long duration, moving slowly down long, low-gradient channels.

Extremely high rainfall in the Eastern Highlands initiated the April floods. But this would normally have created only small-scale flooding, with the flood pulses decreasing downstream through evaporation and infiltration into the underlying beds. On this occasion, however, the arrival of flows from the Highlands coincided with intense rainfall over central and western Queensland, as well as along the Eastern Highlands. New South Wales, meanwhile, having had drought conditions throughout the summer, also experienced intense rain that quickly saturated the ground surface and gave rise to high runoff.

Towns across the region were soon cut off, relying on emergency supplies brought in by air. When a second series of flood pulses arrived from the Eastern Highlands, many towns, such as Charleville on the Warrego River, had to be evacuated. In Charleville alone, the cost of the damage is estimated at over A\$100 million. But the full social and economic implications of the floods are yet to be assessed.

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dependent effects on proliferation of the mesenchymal component, and invokes two different programmes of inappropriate differentiation in the medial epithelial component¹². So at least five different effects of some specificity can be unravelled, and there may well be more. It is likely that the effects of RA in many contexts do not reflect its ability to respecify positional value, as in the limb bud and blastema, nor necessarily its identity as an endogenous signal, but rather that it is a powerful switch which may be useful in analysing developmental mechanisms.

The diversity of effects of RA on cells may now be partly explained by the diversity of RA receptors (RAR) that is being uncovered. The RAR family to date includes α , the original member, β (refs 13,14), γ (refs 15,16) and its relative δ in the urodele blastema¹⁷. Most recently, Mangelsdorf *et al.*¹⁸ have used low-stringency screening to isolate a member of a new family, termed RXR α . In transfection assays for transactivation in cultured cells, the new receptor shows a somewhat different spectrum of activity from that of RAR members. It is activated at higher concentrations of RA, and some synthetic retinoids such as TTNPB, which is active on the limb bud and urodele blastema, have still lower affinities. The difference between RXR α and RAR α is most striking in the ligand-binding E region where there is only 27 per cent identity at the amino-acid level. The authors speculate that the physiological ligand of RXRs may be a retinoid metabolite, with higher affinity than RA itself, that has not yet been identified. The possible response of receptors to high concentrations of a non-physiological lig-

and may be important for interpreting some developmental effects of exogenous RA.

Studies of RAR α , β and γ distribution in the limb bud and mouse embryo by *in situ* hybridization have not so far revealed clear examples of graded distributions, although several interesting features and potential connections with retinoid effects have emerged^{19,20}. Kastner *et al.*²¹ have reported the extension of earlier indications that RAR α undergoes differential splicing to generate isoforms with distinct A regions. There is evidence that in other members of the superfamily, alterations in the A region affect the spectrum of genes activated, and hence some of these isoforms are likely to be functionally distinct²².

By using a combination of library screening and the anchor method of the polymerase chain reaction (PCR), Kastner *et al.* have identified seven different isoforms and provided preliminary evidence for their differential distribution. Two of them lack an A region altogether because of in-frame stop codons, and one contains unspliced intron sequences. The two major isoforms $\gamma 1$ and $\gamma 2$ apparently account for most of the clones after PCR of messenger RNA from

different tissues. Information will doubtless soon be forthcoming on the distribution of the different isoforms, and it will then be quite a challenge to determine their functional role, although the preponderance of $\gamma 1$ in skin has already led to the suggestion that it may mediate the well-known retinoid effects on this tissue²¹. It is clear that the diversity of retinoid receptors is considerably greater than that of other members of the superfamily, and that retinoids are likely to have a variety of unsuspected roles in development and adult physiology.

It seems appropriate to reserve the appellation of morphogen for the graded effects of retinoids on developing and regenerating limbs; and in view of the rather incomplete evidence that such effects are mediated by RA or its relatives *in vivo*, it should be remembered that the increasingly frequent phrase "the morphogen RA" applies for the moment in a pharmacological rather than a physiological sense. □

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HELIOSEISMOLOGY

Shaky clues to solar activity

Douglas Gough

OBSERVATIONS have long revealed that the Sun is an oscillating body. There is an expectation that its oscillation frequencies may change in parallel with the solar cycle, but a lack of long-term data has led to diverse and ambiguous conclusions. Two complementary analyses of different sets of observations, one on page 779 of this issue¹ and the other published in *Nature* last month², now lead towards a consistent view of the dominant structural change that accompanies solar oscillations, and thereby provide pointers towards its possible cause.

The 11-year cyclic variation of magnetic activity, first seen as a change in the sunspot coverage, is the most obvious manifestation of solar variation. Although this cycle has been the object of continued quantitative investigation since the pioneering statistical analysis of Yule³ more than 60 years ago, we still do not understand its origin. An early view⁴ was that it represents an oscillation of the entire Sun, the restoring force for which is provided by the Lorentz force produced by the distortion of a putative global magnetic field, as in a magnetospheric Alfvén wave. Subsequently it became fashionable to consider the cycle as the product of a turbulent dynamo confined solely to the outer region of the Sun, where convection occurs. That idea fell

out of favour when detailed theoretical models failed to explain the most salient features of the observed sunspot distribution. So now attention is being turned to the interface between the convection zone and the radiative interior.

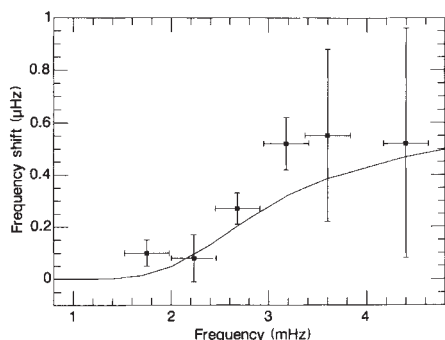
This alone is unlikely to yield the whole story, however, particularly in the light of recent evidence⁵ that the flux of solar neutrinos varies in phase with the sunspot cycle: if this modulation is real, it should encourage one at least to entertain the possibility that it is the source of neutrinos in the solar core that is varying, in contrast to the more popular view (among those who take the modulation seriously) that the cause is neutrino spin flipping induced by magnetic changes in the outer layers of the Sun. Thus we are led back to reconsider the possibility that the original idea of whole-Sun oscillation⁴ contains a germ of the truth.

In view of the success of seismological techniques in probing the interior of the Sun^{6,7}, it is natural to search for temporal variations in the frequencies of acoustic oscillations for clues to the origin of the solar cycle. In the past there have been too few data to enable us to piece together a coherent picture of these frequency changes. Observers have considered it prudent to average their data to subdue the inevitable random measurement

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errors in the results they present. But because this averaging has been carried out in different ways, it has not been possible to compare like with like. The outcome was an apparent disparity among the reports, which is only now being resolved.

The oscillation modes of low degree are



Frequency dependence of the difference $\Delta\nu$ between the cyclic frequencies ν of solar dipole oscillations averaged over data obtained in 1980, 1981 and 1982 near solar maximum and those averaged over 1985, 1986 and 1987 near solar minimum. The data were obtained from whole-disk observations at Izaña, Tenerife, and were analysed by P.L. Pallé, C. Régulo and T. Roca Cortés at the Institute of Astrophysics of the Canaries. The continuous line, which is taken from Fig. 2a of ref. 2, is proportional to M^{-1} , the constant of proportionality having been multiplied by a factor of 1.5 (estimated from the sunspot numbers plotted in Fig. 2 of ref. 1) to take into account the different epochs of observation.

of particular interest, because they penetrate to the Sun's core. Elsworth *et al.*² have presented an analysis of 11 years of data on the average velocity of the solar surface, obtained most recently from a network of up to three observatories placed strategically in different parts of the world so as to achieve almost continuous monitoring. They find that the mean frequencies of oscillation modes of degree $l = 0, 1$ and 2 , averaged over the order of the mode (n) within the frequency range $2.5\text{--}3.5$ mHz, are each modulated in phase with the sunspot cycle with the same peak-to-peak amplitude of about $0.46\text{ }\mu\text{Hz}$. As modes of different degree sample the Sun differently with respect to latitude, this implies (assuming the Sun to be at least axisymmetric) that the cyclic variation is predominantly spherically symmetric. However, these frequency-averaged data cannot tell us at what depth the variation occurs.

Information about the depth dependence is now provided on page 779 of this issue by Libbrecht and Woodard¹, who present data from modes of degree $5\text{--}140$, although from only two years' observations. According to the sunspot numbers, the interval between successive observations encompasses about half of the peak-to-peak amplitude of the modulation in the rising part of the cycle. Thus the frequency increment of about $0.17\text{ }\mu\text{Hz}$ seen

by Libbrecht and Woodard in the 3-mHz modes of lowest degree is more or less consistent with the results from whole-disk data, being indeed roughly half of the peak-to-peak amplitude deduced by Elsworth *et al.* More strikingly, the frequency increment, considered as a function of frequency ν and degree l , is inversely proportional to the inertia, or mass M , of the modes. As Libbrecht and Woodard point out, this implies that the structural variation is concentrated in the very outer layers of the Sun. The acoustic oscillations are modulated mainly by changing conditions in a region in which the waves are evanescent, and not by a change in the cavity within which they propagate.

Although strictly speaking the two reports consider different modes, it is extremely likely that the frequency variations have a common origin. This is borne out by the frequency dependence of dipole modes obtained from the whole-disk data from Izaña, Tenerife (see figure), which exhibit the same inverse proportionality to M . The origin of those variations is, however, yet to be determined. It is evidently associated closely with magnetic activity: not only do the oscillation frequencies vary with the sunspot cycle, but the even component of the degeneracy splitting (a separation of otherwise degenerate frequencies of modes with the same order n and degree l but with different azimuthal order m , caused by a latitudinal variation in the Sun's structure) reported by Libbrecht and Woodard¹ is consistent with the latitudinal variation of activity determined by the brightness of the periphery of the solar image measured by Kuhn *et*

*al.*⁸. The magnitude of the temporal variation in the degeneracy splitting is small compared with the overall frequency shift, confirming the conclusion from the whole-disk data that the structural change is predominantly spherically symmetric.

It is likely that both magnetic activity and structural changes in the superficial layers of the Sun are merely symptoms of a more deeply seated dynamical process. The new oscillation data^{1,2} have not yet revealed that process, and therefore do not answer all of our immediate questions. But they may contain the requisite information, which, once the dominant component of the variation has been accounted for, might emerge from more subtle analysis, which is now under way. What has certainly been provided already, however, is information that should help us to understand the superficial symptoms. That is an important step towards discovering the underlying mechanism that controls the solar cycle. □

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EVOLUTIONARY BIOLOGY

Biological effects of ghosts

Jared M. Diamond

BIG vertebrates have big effects on other species. About one-fifth of the world's bird species and half of its large mammals have become extinct since the late Pleistocene, and many biological features of extant species are likely to have arisen originally as evolutionary responses to them. Recent studies^{1–5} of New Zealand's surviving plants and birds have identified many such legacies of 'ghosts' — in this case, the ghosts of recently extinct large birds. These studies support the growing recognition that modern biology cannot necessarily be understood in terms of modern conditions⁶.

When Europeans reached New Zealand, they found numerous species of plants and small vertebrates, but no terrestrial mammals and no large herbivores or carnivores of any type except humans (Maoris). The subsequent destruction of

New Zealand's native biota by introduced mammals reinforced the view that the biota had failed to evolve defences against large herbivores or carnivores. Closer scrutiny of the flora, however, reveals features known to function elsewhere in the world as anti-herbivore defences: toxic or unpalatable chemicals⁴, mimicry of unpalatable plants or of dead twigs, cryptic coloration, and especially a peculiar growth form termed divarication (branching at a wide angle)^{5,7}.

This form of growth is rare elsewhere in the world but characterizes 10 per cent of woody plant species in New Zealand, where it appears in at least 17 plant families and has thus evolved repeatedly. Divaricating plants have interlaced branches that are difficult to tug or break, tough stems, and few and small leaves on the outside: most large leaves are rele-

gated to the inaccessible interior of the plant, which thus presents an unrewarding woody exterior to herbivores. Divaricating plants also tend to have high nutrient content, as one would expect if divarication evolved to deter large herbivores of a sort absent elsewhere in the world and now extinct in New Zealand. What could those ghost herbivores be?

Pictures courtesy of Richard Holdaway.



Giant predator — reconstruction of Haast's eagle (top) and its huge skull (bottom) compared to that of one of the world's largest living eagles, the wedge-tailed eagle of Australia.

Among the clues are the concentration of divaricating species on fertile alluvial soils and their absence or rarity among plants of cliffs, the alpine zone or canopy epiphytes. It is especially striking that several plant species that are divaricating on the New Zealand mainland are not so on offshore islands, and that nine species divaricating as juveniles cease to be so when they exceed about 3 m in height. As recognized by Atkinson and Greenwood^{5,7}, these clues strongly suggest that divarication evolved as a response to

browsing by New Zealand's moas.

There were about 12 species of these giant, flightless, ostrich-like birds, all of which became extinct soon after arrival of Maoris 1,000 years ago. Preserved moa crops contain the remains of at least 38 plant species³. Weighing up to 200 kg, moas could devour a plant at one bite and must have exerted a considerable selective force. Spines, the usual anti-herbivore device of plants exposed to soft-nosed mammalian browsers elsewhere in the world, would have offered little protection against the heavy, horny head-shields of moas, but divarication would tend to make plants uneconomic fodder.

Abundant fossils show that moas were concentrated on fertile alluvial soils, and were absent from offshore islands and rare in the alpine zone. Moas could not have climbed cliffs or into canopies of trees. The correlation between distribution of moas and of divarication is therefore good. The largest moas were about 3 m tall, nicely matching the height above which plants divaricating only as juveniles cease to be so as adults.

Similarly, among New Zealand's surviving bird species Holdaway² has noted features known to function as antipredator devices elsewhere, despite the absence of large predators among surviving species native to New Zealand. Most of New Zealand's largest extant terrestrial birds — kiwis, kakapo, kea and kaka — are nocturnal or cryptically coloured, or both. The ghosts responsible were two remarkable extinct predatory birds. One was a very large (3 kg) hawk originally described as a harrier but now recognized as the largest of the world's goshawks, a group of bird-hunting specialists. The other was the giant Haast's eagle, at up to 13 kg by far the world's largest predatory bird known from recent times. The eagle would have been the sole New Zealand predator capable of killing an adult moa. One can only speculate what this powerful specialist at attacking tall bipedal prey did when it saw the first arriving Maoris.

Unfortunately, introduced species such as deer and possums browse in ways very different from moas, just as introduced cats and dogs hunt differently from goshawks and eagles. Hence adaptations of New Zealand's plants and small vertebrates to ghosts of large birds failed to protect them against the onslaught of introduced mammals. □

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Bulk betrothal

LAST week Daedalus presented Pairwise, a computer program for the mass arrangement of marriages. Pairwise pairs up a whole population of single individuals, each of whom has a set of 'bond-energies' representing his or her attraction towards each of the possible partners. It optimizes that pairing by iterative annealing. Partners are randomly exchanged between pairs while a fake 'temperature' is slowly reduced, inhibiting further disruption of the stablest established couples. The pairing that finally crystallizes out is thermodynamically stable. Everyone has done as well as possible; divorce and remarriage can never occur.

In today's divorce-ridden world, such mathematical help is sorely needed. Modern populations are socially so mobile that traditional methods of courtship can never examine enough partnerships to reach an optimum pairing. Despite the problem of deducing an individual's bond-energy profile from questionnaires, interviews and so on, Pairwise should be able to do much better. After all, it can marry off whole huge cohorts at a time!

At first, Daedalus will prudently retain several of his program's near-optimal solutions. Each client will be presented with (say) two partners, and an estimate that one of them is 90 per cent sure to be his or her best bet. Even this would eliminate a lot of marital disasters, and Pairwise should get better as its programmers gain experience. But ultimate perfection will always be elusive. One intriguing reason is that, while Pairwise anneals its solution down to an absolute zero, society always has a finite effective temperature. Random 'thermal dissociation' of apparently stable marriages can always happen; more energetic, less stable excited states of the assembly become accessible, and transitions between them can occur.

But what is temperature in this context? The social analogue of molecular energy, says Daedalus, the capacity to make things happen, is money and power. Marriage is at least partly an economic union, and individuals who acquire enough money can in effect buy their way out of it. Those who have power as well are certainly socially 'hotter' than the rest of us, and their relationships are always more labile — as borne out by a steady stream of sensational, highly publicized and expensive divorces. (Other top people, suspects Daedalus, enjoy more discreet forms of marital variety.)

So, says Daedalus, as a society gets hotter, and increasing riches and democracy give its people more power over their own lives, its divorce rate must rise. Strict moralists may prefer that cold political system that gives the people no money and no power — Marxism. David Jones

Problems of 'scientific' whaling

SIR—In a recent Commentary¹, Fukuzo Nagasaki of Japan's Institute of Cetacean Research defended that country's controversial scientific whaling programme. But the programme has been found to be lacking scientific merit by a substantial proportion of the International Whaling Commission's (IWC) scientific committee.

I shall discuss some fundamental issues of the whaling management problem. Nonetheless, there are some errors in Nagasaki's Commentary. He is mistaken in asserting that IWC adopted the moratorium without consulting its scientific committee. Various forms of moratorium were discussed by IWC virtually every year after a 10-year pause was recommended at the United Nations Conference on the Environment in 1972, until the present form was finally adopted in 1982. In that period, the question was frequently referred to the scientific committee. The committee never unanimously recommended a moratorium, which is not surprising since it includes scientists involved in the whaling industry. The proposals for a moratorium did not raise purely scientific questions, but the lack of scientific consensus on nearly all management advice was undoubtedly a key factor in IWC's decision in 1982. IWC agreed to review the effects of that decision by 1990, in part by conducting a "comprehensive assessment" of the status of stocks and management methods.

One of IWC's difficulties is that countries can opt out of its decisions, with the result that the moratorium did not become nominally effective until 1988, 2 years behind schedule. Whaling also continued under a provision allowing countries to issue themselves special permits for scientific research. Japan withdrew its objection to the moratorium in 1988, but joined Iceland and Norway in catching whales under special permit.

Many IWC member countries, aware of past abuses of special permits, were concerned that the moratorium would be undermined. Accordingly, the scientific committee was asked to review research to ensure that it addressed important scientific questions, and that the number of whales killed was not excessive. IWC considered that research should contribute to the future management of whaling, and/or facilitate the comprehensive assessment. Nagasaki fails to mention that IWC, after receiving advice from the scientific committee, adopted resolutions calling on Japan to reconsider its programme because of unresolved scientific controversy about the research, and in the meantime to refrain from catching. Japan has ignored these resolutions.

In the traditional, biological approach to management, attempts were made to

predict the yield from a whale stock by applying estimates of demographic parameters such as pregnancy rates, mortality rates and ages at sexual maturity, to mathematical models specifying the yield as a function of the number of whales in a stock. But this approach is not sufficiently powerful to manage whaling because all demographic parameters have to be estimated to levels of precision that cannot be achieved in reality.

The natural mortality rate is one of the key parameters used for predicting yields. But the natural mortality rate in whales seems to vary with age², as it does in many animals, particularly mammals. Unrecognized age-dependence leads to overestimated average population natural mortality, which in turn leads to optimistic assessments of yield, and hence to catch limits that risk overexploitation and stock collapse.

The main aim of the Japanese research programme is to estimate the age-dependent natural mortality schedule (NMS) for minke whales (the smallest of the large whales, and now the only relatively abundant species) in the Southern Ocean. But Nagasaki is wrong in saying that "anti-whalers" insist that the age-dependent rates be estimated. The existence of age-dependence in mortality means that previous mortality estimates could not be relied on, because samples from commercial catches contain only older animals. But the conclusion drawn by many of Nagasaki's "anti-whalers" was in fact that effort would be better directed towards finding robust methods of management which will not fail if erroneous values for natural mortality and other biological parameters are used.

The Japanese researchers ignored this conclusion and set out in pursuit of an answer to the wrong question. Their programme entails the collection of age data, for which purpose animals are killed to collect waxy earplugs that have annual growth layers. Although it could be useful to know the NMS, it is not agreed that it is either essential, or that estimates with useful precision can be obtained.

The Japanese researchers first suggested using a time series of population age distributions to estimate the NMS. This is impossible³, but this was not conceded until after 2 years of scientific catches had already been taken. What is actually needed is a corresponding time series of rather precise estimates of relative population abundance. Some Japanese researchers seem reluctant to accept analyses showing that these estimates will be cripplingly imprecise because of sampling variability, unless the research were to continue for decades, with total samples of tens of thousands of whales^{3,4}.

If there were no alternative to the traditional approach to management, then refining predictions about the yield by examining specimens could be valuable. But alternative management procedures that do not depend on precise estimates of parameters such as natural mortality are practicable. Various procedures being developed and tested with simulation methods by different groups within IWC's scientific committee perform reasonably well. These alternatives mainly rely on feedback from continual independent monitoring of abundance using sighting surveys, in which whales are counted from ships or aircraft. But the simulations show that the question of population delineation is critical. Whaling is conducted on concentrations of whales, usually on their feeding grounds. Surveys are carried out over much larger areas of ocean, but the whales counted may belong to separate populations, or subpopulations which do not completely mix. Problems arise if catches appropriate for the large area are taken from only a subset of the populations or parts of the area. Satisfactory solutions to these problems are yet to be found.

One of the hopes for the moratorium was that, with whaling in abeyance, a rational discussion could take place on how to overcome deficiencies in whaling management. Instead, scientific whaling has diverted effort into arguing about research programmes which have no prospect of resolving IWC's whaling management problems.

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Myelinated axon origin

SIR—In his recent Scientific Correspondence¹, J. R. Garrett implies that the myelinated axons that I described² in the sympathetic postganglionic nerve following partial denervation of the submandibular gland might not be axons of sympathetic ganglion cells. Because the identity of these processes as axons of superior cervical ganglion cells is fundamental to all of the conclusions in my paper, I would like to respond to Garrett's suggestion.

I presented two pieces of evidence to demonstrate the origin of myelinated axons in the sympathetic postganglionic nerve. First, counting axons in cross-sections of the nerve at multiple sites between the superior cervical ganglion and

the submandibular gland showed that in the experimental animals there is a similar increase in the number of myelinated axons, compared with controls, all along the nerve, consistent with their arising from cells within the ganglion. Second, for both experimental and control animals, applying horseradish peroxidase to the nerve at the site near the gland where a sample was taken for electron microscopy resulted in an almost one-to-one agreement (95 per cent) between the number of axon profiles counted in electron micrographs and the number of retrogradely labelled neurons in the superior cervical ganglion. The source of the 5 per cent of axons that could not be accounted for by this labelling method is unknown and may well include the subpopulation of axons

that Garrett mentions persist after ganglionectomy.

In my experiments this 5 per cent fraction was far too small to explain the large numbers of myelinated axons, more than half the axons in the nerve in some cases, seen after increasing peripheral target size by partial denervation of the gland. Clearly, most of the myelinated axons in the nerve must be axons of superior cervical ganglion cells, which became myelinated as a result of the experimental manipulation.

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Shaking up evolutionary patterns

SIR—My report on parallel gradualistic evolution of Ordovician trilobites¹ kindled a lively discussion (see refs 2, 3). My findings were, of course, not an argument for the universality of gradualism, but suggested that many cases of gradualism are obscured by descriptive biases that generate an impression of punctuation and stasis, irrespective of the real pattern.

Punctuated equilibrium and phyletic gradualism are theoretical ends of a wide spectrum of possible evolutionary patterns. Nature will have found a great many ways of getting from A to B; at issue is the relative frequency of particular patterns. Undeniably, the net changes that the Builth trilobites show in about 3 million years¹ seem very slow on a geneticist's timescale. Nevertheless, the pattern of persistent phyletic evolution resembles the gradualism model far more than stasis. And as finer timescales are resolved the patterns become increasingly dynamic, with frequent short-term reversals of overall trends.

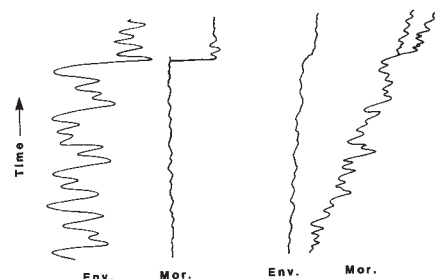
The Builth trilobites lived in a stable, narrowly fluctuating environment¹. One might expect a changing environment to lead to changing morphology, and a stable environment to stable morphology. But over long intervals the opposite may often occur. I suggested¹ that “perhaps . . . gradual phyletic evolution can only be sustained by organisms living in or able to track narrowly fluctuating, slowly changing environments, whereas stasis, almost paradoxically, seems to prevail in more widely-fluctuating, rapidly changing environments”. Evidence for this generalization is growing. It applies mainly to long-term physical variables such as sea level, substrate and climate, rather than ecological ones such as food availability and seasonal differences.

As Maynard Smith said³, “Stasis is a real phenomenon, and a fascinating one”. Morphological stasis lasting millions of years is common in shallow marine shelly

organisms such as bivalves, bryozoans and limulids. Cenozoic marine ostracods and Pleistocene beetles exhibit most stasis during major climatic oscillations.

Perhaps more widely fluctuating environments (on geological rather than ecological timescales) maintain their own kind of stability within wide reflecting boundaries, and selection soon favours lineages with ‘all-purpose’ hard-part morphologies that are relatively inert to each environmental twist and turn (see figure).

The saying “*Plus ça change, plus c'est la même chose*” may be very apt for the relationship between physical environment and phyletic evolution. In conditions that produce complex sedimentary sequences (with many lithological changes and hiatuses) the ability to track preferred environments would be a prime selection pressure, especially on benthic lineages. Whether by selection for habitat tracking or for eurytopy, such lineages might then experience widely fluctuating environments as stable until thresholds are



Model illustrating the hypothesis that persistent phyletic evolution is more characteristic of narrowly fluctuating, slowly changing environments (right), whereas stasis tends to prevail in more widely fluctuating, rapidly changing environments (left). The frequent reversals during gradual evolution are predicted on theoretical grounds². Env, some long-term aspect of environment, such as sea level or temperature. Mor, some aspect of hard-part morphology, such as mean number of ribs in molluscs or trilobites.

reached. As Coope suggested in the case of Pleistocene beetles⁵, strong selection for habitat tracking together with major climatic fluctuations may keep gene pools well stirred, inhibiting speciation and promoting stasis. As a corollary, speciation rates should be highest in the narrowly fluctuating tropics.

In quieter, less dynamic environments species need not be so “generalist” in this sense, and finely tuned, less time-averaged adaptations will have longer-term value. Nevertheless, these lineages would be more sensitive to minor environmental nudges. The nudges themselves would be hard to detect in the geological record, and the inverse of that French saying will appear to be true. Perturbations exceeding rather narrow limits of speed and extent might make such lineages prone to cladogenesis or extinction. Irrespective of probable differences in speciation rates, the model predicts a tendency for more continuous phyletic evolution offshore, and in the tropics generally, and for more stasis in shallow waters and temperate zones. There will, of course, be many exceptions. Plankton, such as planktonic foraminiferans, often show gradualism despite wide climatic oscillations. Presumably these lineages can track (remain within) a narrowly fluctuating environment by simply passive oceanic drifting.

The finding of gradualism in relatively complete stratigraphic sequences and apparent punctuations in less complete ones is normally regarded as an artefact of preservation. But the depositional conditions that affect stratigraphic completeness might themselves sometimes induce such evolutionary patterns.

There may be a correlation between *K*-strategists and phyletic gradualism, and *r*-strategists and stasis⁵. On ecological timescales, *r*-strategists are associated with relatively variable and unpredictable environments. But an ecologist's “unpredictable environment” could be highly predictable over geological timescales.

These hypotheses remain to be tested; we are not yet able to assess accurately the relative frequency of particular evolutionary patterns and the domain of their expected settings⁶. Bridging the gap between the neontological and palaeontological scales of observation may unearth many more evolutionary tales of the unexpected.

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Cloud-reflected radiation

SIR—A recent study of instantaneous solar radiation^{1,2} has shown that anomalously high radiation levels may occur on the Earth's surface during partly cloudy conditions. Values of instantaneous radiation exceeding even extraterrestrial levels are associated with reflections from cloud edges to positions which otherwise have a clear view to the Sun. Here we report measurements of increased ultraviolet radiation associated with cloud reflection.

Conventional integrated hourly or daily solar radiation measurements inherently involve the averaging of successive instantaneous events and the consequent loss of the short-term behaviour such as the bimodal clear/cloudy radiation pattern¹. Therefore, the full extent of radiation increases resulting from cloud reflection can only be appreciated on the basis of instantaneous radiation measurements. Figure 1 shows measurements of instantaneous global radiation during a clear-cloudy transition. The increase in radiation level just before the rapid drop results from additional radiation reflected from the cloud edge before the cloud passes between the solar sphere and the pyranometer. Radiation levels of up to 1,780 W m⁻² have been recorded in this manner. The existence of such high terrestrial radiation values in excess of extraterrestrial levels (1,370 W m⁻²) may seem surprising at first. But the principle of conservation of energy is not violated as the high radiation values constitute local atmospheric concentrations of the solar radiation.

Measurements of instantaneous ultraviolet radiation during periods of elevated radiation levels attributable to cloud reflection have been carried out. In Fig. 2, values of the ratio of cloud-reflected ultraviolet radiation to clear-sky ultraviolet radiation at wavelengths of 350 and 400 nm respectively, are plotted as a function of the corresponding ratio for broadband solar radiation. The results shown in Fig. 2 are for a 3-hour interval and are typical of measurements taken over a 1-year period. In all cases the increase in

ultraviolet radiation due to cloud reflection is approximately linearly related to the corresponding increase in broadband solar radiation. As seen from Fig. 2,

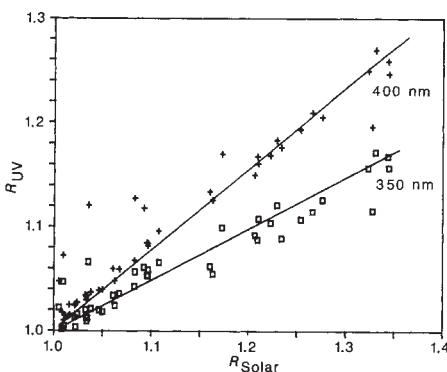


FIG. 2 Correlation between cloud reflection enhanced ultraviolet at 350 and 400 nm and broadband solar radiation. R_{UV} and R_{Solar} are, respectively, the ratios of cloud-reflected ultraviolet and solar radiation to the corresponding clear-sky values. Broadband solar radiation measurements were made using a Kipp and Zonen CM10 pyranometer with thermal response correction³.

the ultraviolet radiation concentration decreases with decreasing wavelength. This behaviour results from scattering at cloud water droplets which becomes more forward directed with decreasing wavelength⁴, resulting in greater ultraviolet cloud transmittance and less radiation reflection due to multiple scattering. Although the increase in the level of ultraviolet radiation due to cloud reflection is less than that for broad-band solar radia-

tion, the increases measured are of significance in view of the predicted increase in cloud cover associated with the build-up of greenhouse gases and the importance of ultraviolet radiation to biological processes.

The radiation concentration strongly depends on the cloud type. Large cumulus clouds with cloud edges several kilometres high are likely to produce strong radiation concentrations. Hence, the intensity of the radiation concentration will depend on the clouds and climate of the location of investigation⁵. For comparison, in tropical Townsville, Australia, peak radiation values as high as 2,020 W m⁻² have been measured with a photovoltaic pyranometer. The effect of atmospheric radiation concentrations may be more significant than is widely believed.

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More false-positive problems

SIR—Sarkar and Sommer have reported in Scientific Correspondence¹ the use of ultraviolet irradiation as a sterilization technique for the control of contamination in the polymerase chain reaction (PCR), but their procedure does not deal with all the problems. First, they introduce true target DNA and *Taq* DNA polymerase after irradiation, which will produce the same level of sporadic

false-positive PCR signals even though all the contaminating carryover molecules in the other PCR reagents are sterilized. Second, their conclusion that the oligonucleotide primers for the PCR retain their full functional integrity after irradiation is not warranted. Primer damage which leads to a compromise in signal sensitivity can be evaluated only when the PCR amplification

is limited so that the concentration of product is maintained well below PCR plateau concentrations. Finally, the critical nature of the size and sequence specificity of the PCR product being inactivated was not addressed. Here, we elaborate only on the last point.

Pyrimidine dimers undoubtedly contribute to ultraviolet-induced sterilization by functioning as termination sites during the extension reactions of the

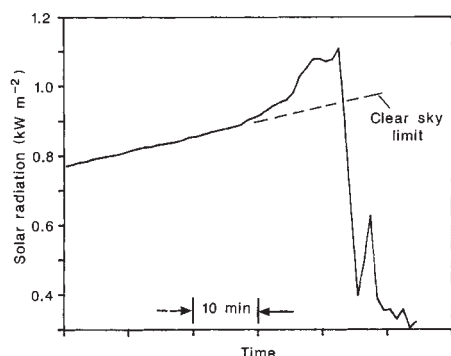
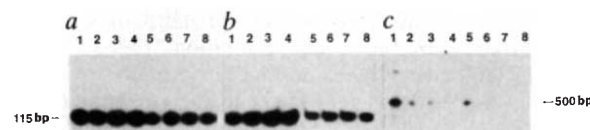


FIG. 1 Variation of incident solar radiation during a clear-cloudy transition.



Effect of PCR product length on UV sterilization of PCR products. Two PCR products were examined: a 115mer (Ou *et al.*⁶) and a 500mer (kit N801-0055, Perkin-Elmer Cetus). PCR templates were irradiated for 0 min (lanes 1 and 5); 5 min (lanes 2 and 6); 20 min (lanes 3 and 7); 30 min (lanes 4 and 8), amplified for 30 cycles and analysed by PAGE and autoradiography. In panels a (115mer template), b (plasmid template) and c (λ DNA template). Lanes 1-4 and 5-8 correspond to 10⁵ and 10³ copies of PCR template molecules, respectively. Detection sensitivity with our PCR procedure is at least 100 copies. Experimental details available from authors upon request.

PCR procedure. Only a fraction of all pyrimidines within a DNA strand form dimers and, as dimers are both made and broken by ultraviolet exposure, this fraction establishes a steady-state level which varies with the irradiation wavelength, the type of pyrimidine dimer and the nucleotide sequences next to the dimer site^{2,3}. At steady-state, an upper limit for the number of all dimer defects in a long, irradiated DNA molecule is less than 0.065 defects per base pair⁴. Non-dimer photodamage (for example non-cyclobutane-type pyrimidine adducts, thymine glycols, interstrand and intra-strand DNA-DNA crosslinks and DNA strand breaks) can also be termination sites for *Taq* DNA polymerase. The number of these sites should be at least equivalent to the number of dimer sites⁵.

If these defects (0.13 total defects per base pair) are randomly distributed throughout a DNA molecule, a 500-base-long oligonucleotide would have an average of 32 damaged sites. In a population of 10⁵ DNA strands containing an average of 32 termination sites per strand, there is a minute probability that any strand is without at least one termination site. By comparison, 100-base-long PCR products will have an average of only 6 modified sites per strand at this same defect density. In a population of 10⁵ molecules with just six average termination sites per strand, a statistically large number of molecules will contain no modified sites and therefore are not sterilized.

Furthermore, to be effective for PCR sterilization, the photoinduced defects should be in the sequence region bounded by the 3' ends of the PCR primers. These considerations predict that ultraviolet irradiation would be effective for long but not for short PCR products. Our experiments demonstrate that this is the case.

We irradiated targets with either 254, 300 or a combination of 254 and 300-nm bulbs. After irradiation, the remaining PCR reaction components were added to each tube and amplification was performed. As shown in the figure, a 115mer PCR product is efficiently generated from as little as 10³ target molecules that had been irradiated with a combined 254 and 300 nm exposure independent of whether the target is a synthetic 115mer or an 11-kb plasmid. Irradiation of linearized λ DNA from which a 500mer PCR product is made results in 10³ copies of the λ being sterilized with the combined 254/300 nm procedure. But 10⁵ copies of the λ -DNA target show an exposure dependent sterilization. Only 30 minutes of exposure is sufficient to reduce the PCR signal below the sensitivity level of the gel assay.

These data imply that caution is needed when applying direct ultraviolet irradiation as a sterilization process in a pre-amplification mode. Both the length of the PCR product and its internal sequence

must be considered. The 115mer we used was chosen because it has a very high fraction of adjacent thymines between the primer regions of the PCR product. But the distribution of thymines is such that most of the potential pyrimidine dimers will occur on one strand of the product. This type of distribution must also be taken into account when considering ultraviolet sterilization techniques.

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Hiccup for hiccups

SIR—Fuller¹ suggests that hiccups represent an essential intrauterine reflex to allow vigorous exercise of fetal inspiratory musculature in preparation for extra-uterine breathing. But this idea has not been seriously advanced since 1902. Respiratory views of hiccup function fell from favour as gastrointestinal rather than respiratory events were found to promote hiccups (for example, ref. 2).

Contrary to Fuller's claim, hiccups differ from developmental reflexes in that they do not entail reliable muscle or nerve actions (one or both hemidiaphragms may be involved, or accessory muscles of respiration, and possible afferents are legion); they are never reliably elicited; and they do not cease at a defined developmental stage. The argument that hiccups affect all humans and respect a developmental profile is equally valid for bed-wetting and drooling. These have no function but reflect immaturity — or pathological disruption — of neuronal control systems. Even cardiac arrhythmias are universal. Arrhythmias, like hiccups, may result from an imbalance of neuronal excitation and inhibition. Indeed, both heart block and hiccups are provoked by high vagal tone, and the two may occur together^{3,4}. So hiccups could be good for nothing.

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Treat with care

SIR—The association recently found by Gardner *et al.*¹ between paternal exposure to ionizing radiation before conception and childhood leukaemia in offspring has raised considerable concern and pressure to confirm or deny the association. But radiation is only one of the possible risk factors to which men working in an industrial/chemical environment such as that of nuclear-fuel reprocessing might be exposed. Regrettably, the current state of understanding of the underlying theoretical basis for the action of ionizing radiation on living organisms does not allow opinion to be expressed one way or the other on the validity of a causal association with radiation.

Evans, in his News and Views article², has outlined in some detail the reasons for scepticism concerning the possibility that the association reported by Gardner *et al.* is casual. And in Scientific Correspondence last week³, Nomura, whose experiments with mice⁴ have been widely quoted, pointed out the factors that may confound extrapolation of his results to help explain a causal basis for the data of Gardner *et al.*

Childhood leukaemia is not necessarily related to any form of leukaemia in adulthood, and probably has origins at the embryo stage, as shown by the increased risk of this disease in children irradiated *in utero*⁵. In mice, no distinction can be made between such neoplastic disease and adult neoplasia, and there is no authenticated model for human childhood leukaemia in any animal.

For these reasons, and also because of the marked strain differences in radiation-induced neoplasia in animals and the scale of experiments required to detect effects at low doses and low-dose rates, it would be extremely difficult to devise a reliable test of the Gardner hypothesis using animals.

But Gardner *et al.* have put forward a clear-cut hypothesis relating doses before conception to a specified increase in the risk of childhood leukaemia. Although this is obviously not a simple task, the hypothesis should be tested on other populations of workers who are exposed to radiation but not to the other hazards of chemical processing, such as organic solvents (some of which are known to be leukaemogens), before people begin large-scale animal experimentation.

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Dealing with the disaster

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The Legacy of Chernobyl. By Zhores A. Medvedev. Blackwell: 1990. Pp.352. £19.95, \$29.95.

ANYONE who has worked in accident investigation will know how difficult it is to establish the facts about even simple accidents. Nothing about the Chernobyl accident was simple. Its immediate cause was a test to assess the effectiveness of a new system for extracting emergency power from a turbo-alternator as it loses speed following a failure of the steam supply. The test was badly conducted, but would not have led to an accident in a well-designed power plant. The Chernobyl plant depended for its safe operation on various administrative rules. In the approach to the test, the rules were severely infringed and the test started with the reactor in a seriously unstable condition. In a few seconds, it reached 100 times its rated power, the fuel fragmented and caused a steam explosion (and probably a hydrogen explosion) which lifted the top shield of the reactor and set fire to the graphite moderator. Significant contamination was spread to much of the Soviet Union and most of Europe. The problems of dealing with the accident and assessing and, where necessary, alleviating the consequences have been immense. They still continue.

The most impressive feature of Medvedev's book is the extent of its coverage of the technical and popular reporting of the events. A prodigious amount of work has been done. Medvedev does not pretend to seek an unbiased position. He is, understandably, unsympathetic to the Soviet system and disinclined to accept official views. That makes the book more readable, but sometimes leads the author into unnecessary controversy. Medvedev is obsessed by the original test, which he sees as unnecessary, or at best ill-timed. To a large extent, that is because he fails to understand its purpose.

Medvedev starts his book with an account of the reactor and its accident. Most of this is clear and readable. In places, however, his lack of understanding leads him into confusion. The idea of a local insertion of control rods round a damaged fuel channel to isolate it "without shutting down the whole reactor" is an example. The account of the fire-fighting is graphic and includes a mass of circumstantial detail. It is built up from many separate stories and may owe something to dramatic licence. It includes the bizarre suggestion, accepted as authoritative by

Medvedev, that a "vacuum" formed beneath the materials dropped from helicopters into the reactor structure. The "vast bulk" of these materials then crashed down, throwing out "a huge

the repeated use of the 'micromillimetre' in place of the micrometre. Medvedev comments unfavourably on the difference between British and US estimates of the number of future cancers to be attributed to the accident. In fact, he shows that the estimates rarely differ by more than a factor of two, well within the uncertainties of the necessary environmental modelling. Nevertheless, the chapters provide an impressive account of the deposition of fission products and clearly show the difficulties of summarizing the very non-

Poppertoto

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

Aftermath of catastrophe — checking radioactive levels in Kiev, May 1986.

amount of radioactive dust".

The chapters on the environmental and agricultural impacts give a chilling indication of the problems facing the Soviet authorities and indicate why it is likely that they were not able to take widespread effective action. It was clear at the International Atomic Energy Agency meeting in 1986 that the Soviet account of emergency action in the environment was a good deal tidier than the reality, but few people felt critical. The problems were so much worse than those in Europe, where the organization was far from impeccable, that the mood was one of sympathy rather than criticism. These chapters contain many numerical statements, but few quantitative interpretations or judgments. Stories of farm animals deformed at birth are quoted without comment on the likelihood of a causal link to radiation — an unexpected omission for a man who describes himself as a "biologist with a background in agriculture and radiobiology".

The chapters on health impacts are also very qualitative, despite many numerical details. Confidence is not improved by the specification of concentrations in air in units of curies per square kilometres (the error may be in the source material) and

uniform pattern of that distribution.

Surprisingly, the book is at its weakest when dealing with the biological effects of the exposures and with the policy of radiation protection. On several occasions, Medvedev quotes advice from the International Commission on Radiological Protection. Most of this material is distorted and some of it is pure invention. The commission does not say that "If the projections indicate a possible dose of 0.75 Gy (75 rem), the evacuation of the population is obligatory". It does recommend an annual dose limit for workers in normal conditions of 50 mSv in a year (now under review), but certainly not a lifetime limit of 250 mSv. Medvedev also makes much of the dangers of "hot particles". These are small particles of concentrated radioactive material. Many years ago, there was alarm lest the very high local doses to the lung from inhaled particles might cause a higher risk of subsequent cancer than would more diffuse doses. Experimental studies have since shown that the reverse is true: it is now clear that high local doses cause the death of cells and reduce the chances of cancer relative to the diffuse doses.

This is a difficult book to sum up. The author makes an excellent job of indicating

the enormous problems posed by the accident and the serious long-term effects on both health and nuclear power. It is flawed by a lack of quantified interpretation and by many errors, some due to ignorance and some to carelessness. I am sure that Medvedev knows that the water cooling the condensers of a boiling water reactor does not pass through the core and that iodine isotopes do not decay by fis-

sion, but he has allowed these absurdities to remain in the text. I can recommend the book to those who want a general account of Chernobyl and its aftermath and who are not too worried about the accuracy of detail, but I cannot recommend it as an authoritative source of information. □

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American pie

Tim Lincoln

The Newtonian Casino. By Thomas A. Bass. Longman: 1990. Pp.329. £12.95*.

IN THE early years of their statehood, the economies of California and its neighbour Nevada rested on digging gold and silver out of the ground. In 1931, with the legalization of gambling, Nevada decided on the more straightforward strategy of digging them out of people's pockets as well, not least the pockets of Californians. The high life of casino owners in Las Vegas and in Reno, its smaller northern counterpart, was (and is) in part paid for by the losses at their tables of the citizens of Los Angeles and San Francisco.

Thomas Bass's book is an insider's account of how, during the late 1970s, a bunch of free-wheeling young physicists centred on the University of California at Santa Cruz attempted to recoup some of that money. Their dream was to build for themselves a world above workaday considerations; their way of realizing it, like that of many before them, was to beat the house at roulette. It is an extraordinary story that is believable only because, true to life, it rambles somewhat, and because the dénouement is one which no publisher of fiction would have tolerated.

Luck isn't much help in winning at roulette, except in the short term, nor are mathematical systems, which require a bank of infinite size and a casino that doesn't set an upper limit on bets. Instead the Santa Cruz group set itself the task of constructing a prediction system based on the laws of physics. If enough is known about the forces acting on the ball and rotor of a particular roulette wheel, the ball's final resting place can be estimated with enough certainty to give the gambler (with this system a gambler no more) a comfortable advantage over the house. The problem then, nontrivial as it turned out, was to write a computer program of sufficient sophistication to take account of all the variables; to build the hardware to run it; and to devise a method of communication in which the prediction could be transmitted from one person

taking the data to another placing the bets. There was also the small matter of staying cool in casinos, places not renowned for their love of winners, when wired up with roulette-beating electronic equipment.

Two things made the physicists' assault on Nevada different from the attempts of those who had gone before them. One was that greed was tempered not only by idealism but by a thoroughly businesslike attitude. A company was formed, Eudaemonic Enterprises, (eudaemia being the spirit of rationality), the company's object being to create a pie from the winnings in which everyone who had contributed to the project would share. The other was miniaturization of the equipment, made possible only by the advent of the micro-processor in 1970; Silicon Valley, of course, was on the doorstep.

That the entire system ended up being fitted into three shoes — two worn by the data-taker, the other by the person laying the bets — was a triumph of perseverance and ingenuity. That their work on the

project gave some of those involved a head start in the study of the new science of chaos, and ultimately academic respectability, was serendipitous.

Bass's chronicle is one of breakthrough and snafu. He is strong on the technical details, reticent about disagreements (surely there were more than he mentions). But they must have been heady years, the slog of putting together a working system and the frustrations of getting it to operate in casinos being punctuated most notably by crazy Halloween parties.

Behind the fun and games there lies an economic parable of the decade just passed. Half-way through the book, Wozniak and Jobs, the founders of Apple computers, make a fleeting appearance as the exemplars of dope-smoking, long-haired technological genius. At that point, one of the cast remarks: "As Xerox and IBM move in to package everything, I wonder if there's still going to be room for the hackers. That's where this country is still way ahead of the Japanese [who] don't have any place. . . for misfit hackers. But these are the people coming up with the truly creative ideas." When, ten years ago, Americans elected Ronald Reagan president, they announced that conservatism — short hair and much else besides — was back in fashion. Misfits aren't often found in a Brooks Brothers' suit. That might have been the day when, for innovation in the United States, the music started to die. □

Tim Lincoln is News and Views editor of Nature.



Species in danger — Texas poppy-mallow (*Callirhoe scabruscula*). Protecting the interests of threatened species, the World Wildlife Fund has recently issued *The Official Guide to Endangered Species of North America*. This two-volume set — with a supplementary photo locator, listing sources for photographs of threatened or endangered plants and animals — provides updated population figures, recovery efforts and habitat descriptions for 515 listed species of plants and animals. Published by Beacham Publishing, Inc., 2100 S Street NW, Washington, DC 20008, USA, \$195.

* Published four years ago in the United States by Houghton Mifflin under the title *The Eudaemonic Pie*.

Making sense of the past

Colin Renfrew

A History of Archaeological Thought. By Bruce G. Trigger. Cambridge University Press: 1990. Pp. 500. £37.50, \$59.50.

THE late Glyn Daniel was the first to realize that the really important advances in archaeology were not the great discoveries, the spectacular excavations or the gold of the pharaohs. The real task of archaeology is not the unearthing of relics, although that too has its place, but the undertaking of making sense of the past. This view was already implicit in his *A Hundred Years of Archaeology* (Duckworth, London, 1950), the first, and until now the only satisfactory history of the subject. It found its most coherent expression in his *The Idea of Prehistory* (Watts, London, 1962), which remains the most coherent and attractive introduction to archaeology-as-thought rather than archaeology-as-dug.

It is the great merit of Bruce Trigger's *A History of Archaeological Thought* that it builds on the perception of Daniel that the true history of archaeology is the history of ideas, using and reflecting the great self-awareness in matters of theory and method which came to the subject with the emergence of the 'new archaeology' around the time that the first edition of *The Idea of Prehistory* was published. Of course the new archaeology ensures that it remains a materialist discipline. But with its inception came what the late David Clarke aptly termed "the loss of innocence", and innocence once lost can never be regained.

Trigger's volume has several merits which establish it without question as the only adequate successor to date to Daniel's *A Hundred Years of Archaeology*. In the first place, Trigger is well read in the archaeology of Europe as well as of North America. He is well aware that the upsurge of archaeological theory in the United States over the past 25 years does not automatically establish North America as the only place where interesting theoretical developments are taking place, or even the most important. He does not suffer from that intellectual myopia which afflicts in this respect so many of his North American colleagues. This is the first book which sets out to review the theoretical developments of our time in a manner beyond the parochial. Indeed he makes a real effort here — perhaps not entirely successful — to go beyond the confines of the English-speaking world: there is a serious attempt to bring Soviet archaeology into the discussion, as well as that of Japan, Africa, New Zealand and Australia. The work is up-to-date (to 1986 or 1987) and Trigger is exceedingly well read: the bibliography contains

more than 1,300 titles.

Not surprisingly, Trigger's new book reflects in large measure the preoccupations of his earlier writings. Gordon Childe is given pride of place among archaeological thinkers, reflecting the interests developed in Trigger's *Gordon Childe: Revolutions in Archaeology* (Thames and Hudson, 1980). I would certainly not quarrel with that assessment, and the discussion of Childe here (as well as that of Grahame Clark) is particularly satisfying. Less satisfying in my own evaluation is the bias already evident in his *Time and Tradition: Essays in Archaeological Interpretation* (Edinburgh University Press, 1978), where Trigger contrasted the "nomothetic" tendencies, which he saw as characteristic of the new archaeology, with the "idiographic" approach of the historical tradition.

This dichotomy underlies Trigger's treatment of the archaeological thinking of the past 25 years. He is critical of the constellation of ideas which he identifies by linking the 'positivist' aspects of the new archaeology with what he regards as the 'neo-evolutionism' of US writers in recent years. He does not respond by embracing wholeheartedly the position of the self-proclaimed "post-processual" school which has grown up in opposition to the processual outlook of the new archaeology, for he sees the dangers inherent in the extreme relativist position which they have not been able to avoid. But it is not quite clear what he sees as an appropriate alternative. His approach is clearly a particularist one, but he seems dimly aware that explanation usually entails some elements of generalization. For me this is the weakness in Trigger's position, and it is one which colours his entire approach, making him (in my view) undervalue the originality and the lasting value of the contributions made by Lewis Binford and some of his colleagues. Of course Trigger does not dismiss these — his intention is to offer a balanced view, and many of his criticisms are reasonable ones. But generalization gets a bad press in this book, frequently being lumped together with the supposed excesses of 'neo-evolutionist' thought, whose significance for US archaeology he considerably exaggerates. The breadth of Trigger's reading, and the catholicity — pluralism almost — of his position means that this bias does not fatally damage his argument. But as a critique of current positions it is hardly a dispassionate treatment.

More obviously irritating is the curi-

ously simplistic historicist treatment of the relationship between recent historical events, such as the dropping of the atom bomb or the Vietnam war, and the archaeological thought of the time. That the *Zeitgeist* should influence writers and thinkers comes as no surprise, and Trigger's comments on the outlook and social milieu of individuals, such as Sir Arthur Evans, are perhaps reasonable enough. But time and again snap judgements about the spirit of the time are used to 'explain' dominant trends in archaeological thought. For instance (page 289): "The neo-evolutionism that developed in the United States in the 1960s was yet another attempt by anthropologists living in a politically dominant country to 'naturalize' their situation by demonstrating it to be the inevitable outcome of an evolutionary process..."; or (page 323): "While the origin of ideas has no necessary bearing on whether or not they are correct, it is fairly obvious that the high-level evolutionary theories that guided the interpretation of archaeological evidence in the 1970s reflected a serious and prolonged economic, political and social crisis in which the interests of the dominant middle classes were perceived as deeply threatened." This seems to me pure claptrap. For one who would eschew the generalizing approaches of processual archaeology, Trigger seems remarkably ready to generalize about these highly nebulous linkages. As hypotheses they might be worth investigating (if one could devise a strategy for doing so); as a serious commentary on recent archaeological thought they appear to me of a superficiality which contrasts markedly with the careful and scholarly treatment of the book as a whole. There are many such examples of this politico-philosophical historicism. But the reader (and perhaps the author, in a second edition) can readily discount them. They may be an irritation, but they do not seriously damage what is in most other ways a major and frequently very satisfying contribution to the field.

This is the only good and up-to-date history of archaeology now available, and a worthy successor to those mentioned earlier. The author has succeeded admirably in accomplishing what is almost a world survey, presenting a wide variety of ideas both lucidly and accurately. It is a work which all thinking archaeologists will wish to have on their shelves.

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■ Cambridge University Press has recently published *The Archaeology of Human Origins*, a collection of the most important and influential papers written by the late Glyn Isaac. Edited by Isaac's wife and collaborator Barbara Isaac, price is £37.50, \$59.50.

Eightfold ways

Peter J. Smith

Catastrophism: Systems of Earth History.

By Richard Huggett. Arnold: 1990. Pp. 246. £35. Distributed by Routledge in the United States, \$44.95.

THE conflict during the early nineteenth century between the proponents of catastrophism and uniformitarianism is one of the more enduring part-myths in the history of geology. In its more extreme, picture-book form it pits irredeemable baddies (the catastrophists) against impeccable goodies (the uniformitarians), with ultimate victory going to the white knight Sir Charles Lyell acting, in part, under the historical influence of James Hutton. That there were differing views on the nature and causes of geological processes and events is undoubtedly true; but as the revisionists, led by Tony Hallam and Stephen Jay Gould, have so ably shown over the past decade, shades of opinion were more complex than the Henty school of historians would have had us believe. Huggett's main achievement is not only to reinforce the revisionist line in respect of the early geologists but to demonstrate that the uniformitarianist rhetoric of the 'victors' has been at odds with the reality of much geological thought since at least the late nineteenth century.

Part of the barrier to understanding here has been the inability or unwillingness of working geologists to become conversant with the precise ground rules for debate, which is no doubt why Huggett takes the early part of his book to define terminology and spell out the fundamental philosophical concepts involved. Essential though this may seem, it is a

courageous move all the same, for, being akin to insisting that the learning of tables is a necessary prerequisite for arithmetic operations, it involves the severe risk of turning geologists off. They would be wise to master this section, however, not only because Huggett goes on to clarify the nature of the work of individual geologists in terms of classification systems set up therein, but because the failure hitherto to assimilate the fine philosophical distinctions explains just why it is that (to adapt an adage more usually applied to economists) whenever six of today's Earth scientists are gathered together there will emerge seven different definitions of uniformitarianism.

The fact is that Lyell proposed not one but four distinct uniformities — of law, of process (actualism), of rate (gradualism) and of state (steady-statism) — the first two of which are methodological principles to which most geologists have hitherto subscribed and the last two of which are substantive claims about the Universe that Lyell partially disguised as methodological principles. All have their attendant confusions. The uniformity of law could be regarded as tautological, although that is the least of a geologist's worries. More seriously, the uniformity of rate raises the vexed issue of the definition of rate in the context of periodic, cyclical and episodic change and, not least, of the potential of uniform-rate systems to generate catastrophic modes of action. The conclusions reached here then have implications for the precise meaning of the uniformities of process and state.

The picture is further confused by the fact that catastrophists also subscribed to the principle of the uniformity of law and, in part, to that of the uniformity of process. On the other hand, in their early manifestation they were also deists, semi-deists or theists, but then lyellian uniformitarianists were deists too. And irrespective of by what or whom catastrophes are caused, and whether they are externally imposed (by either natural or divine process) or the consequence of internal crises, just how are 'catastrophes' to be defined anyway, given their wide range of spatial and secular scales?

Huggett has done a truly magnificent job in separating out the many conceptual strands that weave their way through the history of geology, ending up with two eightfold classification schemes (one each for the inorganic and organic worlds) into which he proceeds to fit theories of life and Earth old and new. The result is a brief history of geological thought that demonstrates convincingly that, notwithstanding uniformitarianist claims to the contrary, catastrophism of various forms and scales has almost always been an essential component of geological theory. This also includes geographical theory, incidentally, for Huggett has chosen to use

more examples from his own sphere of the Earth sciences than the average geological reader will be used to.

This wider frame of reference is all to the good, although I rather suspect that Huggett's geographical background may have led him to underestimate somewhat the degree to which modern geologists have been willing to accommodate natural catastrophism. There is, in fact, a curious paradox here that requires further examination. It is undeniably true, as Huggett says, that geologists are so wedded to the *idea* of what they understand to be uniformitarianism that in order to reconcile this devotion with modern geological theory they have had to resort ever more desperately to such ludicrous terms as 'episodic uniformitarianism' and even 'catastrophic uniformitarianism'. On the other hand, one can only look with astonishment at the sheer number of Earth scientists who have been able to accept, apparently without turning a hair, the extraterrestrial-catastrophist explanation of the iridium layer (and hence, by extension, the mass extinctions) at the Cretaceous-Tertiary boundary. I don't recall reading over the past decade a single objection to the asteroid impact hypothesis on the purely philosophical grounds that such an impact could be interpreted as non-uniformitarianist. Objections there have been plenty, but most conspicuously from geologists peddling an alternative explanation that itself involves catastrophism of a sort. Why are professed and actual beliefs so ill-matched? □

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New Journals

This year *Nature's* annual new journals review supplement will appear in the issue of 11 October. Publishers and learned societies are invited to submit journals for review, taking note of the following criteria:

- Journals which first appeared after June 1988, and which issued at least four separate numbers by the end of April 1990, will be considered for review. The deadline for submission is the end of June.
- Journals covering any aspect of science are eligible, although those dealing with clinical medicine, engineering and pure mathematics are excluded, as are publications of abstracts.
- Frequency of publication must be at least three times a year.
- The main language used must be English. Translation journals in English are eligible.

When submitting journals for review, please send at least four different issues (the first, the most recent and any two others) of each title as soon as possible to: Book Review Editor, *Nature*, 4 Little Essex Street, London WC2R 3LF, UK or 1137 National Press Building, Washington DC 20045, USA. □

New in paperback

- *The Fate of the Forest*, by S. Hecht and A. Cockburn, tells the story of the delusions and greed that have shaped the Amazon's history. Subtitled *Developers, Destroyers and Defenders of the Amazon*, it was reviewed in *Nature* by M. Fox (342, 313; 1989). Penguin, price £5.99.
- Johns Hopkins University Press has just issued in paperback *Back to Nature: The Arcadian Myth in Urban America*, by P. J. Schmitt, originally published by Oxford University Press in 1969. Schmitt describes how the American urban middle class became involved with the countryside from the turn of the century to just after World War I — its search for virtue in nature. Price is \$12.95.
- New in paperback from Princeton University Press is *Controversy in Victorian Geology* by J. A. Secord. The book provides a new interpretation of the Cambrian-Silurian debate, relating the dispute to the social background of British geology at the time. Price, \$16.95.
- A delightful facsimile edition of N. Tinbergen's classic *Social Behaviour in Animals* has been issued by Chapman and Hall. The book is based on Tinbergen's own research from which emerged the discipline of ethology — the scientific study of animal behaviour. Originally published in 1953, the book contains the author's original sketches and photographs. Price is £12.95.

Solar-cycle effects on solar oscillation frequencies

K. G. Libbrecht & M. F. Woodard

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Measurements of solar oscillations taken in 1986 and 1988 show systematic changes in the Sun's acoustic-mode frequencies of the order of 1 part in 10,000. These data reveal that the frequency shifts are the result of latitude-dependent changes in the structure of the Sun which are correlated with the Sun's magnetic-activity cycle.

JUST over a decade ago, precise observations of the Doppler shift of integrated sunlight (that is, not imaged) demonstrated that the Sun exhibited globally coherent five-minute oscillations^{1,2}. Sharply peaked features were found in power spectral analyses of the integrated Doppler measurements, and these features were interpreted as global normal modes of the Sun. These modes, called p-modes because pressure is the dominant restoring force, are small amplitude ($<20 \text{ cm s}^{-1}$ in surface velocity) acoustic oscillation modes, which are probably excited by turbulent convection in the Sun's outer layers^{3,4}.

Subsequent observations of Doppler and intensity images of the Sun showed that at least several hundred thousand globally

coherent p-modes are excited to observable amplitudes. The perturbation of a scalar quantity, for example pressure, resulting from the excitation of a single mode can be characterized by three 'quantum' numbers, n , l and m (assuming a slowly rotating Sun): $\delta p_{nlm}(r, \theta, \phi, t) = \text{Re} [\delta p_{nl}(r) Y_l^m(\theta, \phi) e^{i2\pi\nu_{nlm}t}]$, where $\delta p_{nl}(r)$ is a radial wavefunction with n nodes, $Y_l^m(\theta, \phi)$ is a spherical harmonic of degree l and order m , and ν_{nlm} is the frequency of the mode. Because different modes are described by different wavefunctions, observations of p-mode frequencies can be used as a probe of the structure of the solar interior, as has been discussed in several recent review articles⁵⁻⁷.

Ever since the discovery of global p-modes in the Sun, it has been expected that solar-cycle changes in the Sun's interior structure would result in observable changes in the mode frequencies, and therefore provide a possible indirect probe of a deep-seated magnetic dynamo. Although there are indications in recent low- l data that mode frequencies near 3 mHz are perhaps $\sim 0.4 \mu\text{Hz}$ greater at solar maximum than at solar minimum, the signal-to-noise ratio in the published observations is not very high, and there is some disagreement between different observations^{8,9}. Measurements of intermediate- l mode frequencies ($5 \leq l \leq 100$) have also been used to search for

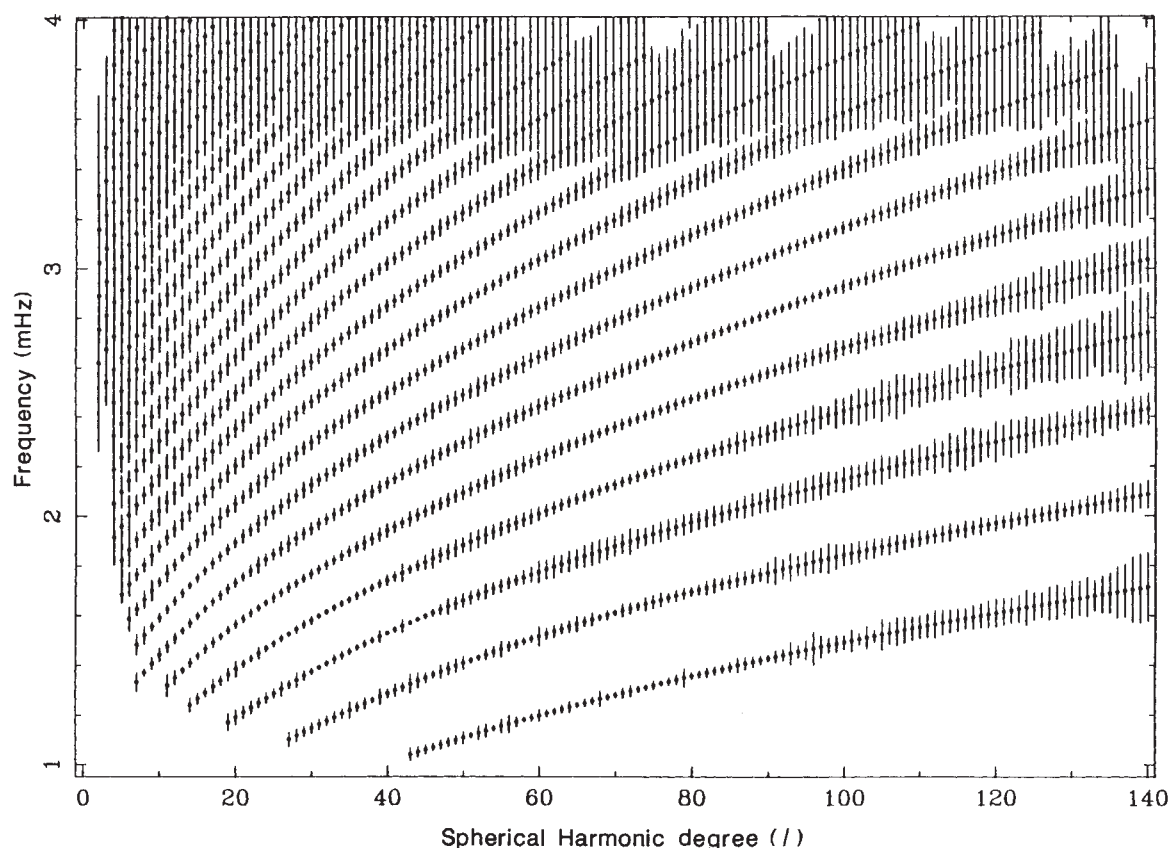


FIG. 1 Schematic plot showing the observed solar p-mode frequency measurements, where the usual 1σ error bars have been magnified by a

factor of 1,000. Each ridge contains modes with a fixed number of radial nodes n , with the lowest frequency ridge having $n=1$.

TABLE 1 Splitting coefficients (nHz) at 3 mHz

	1986	1988	1988–1986
$\Delta\nu$	—	—	186.0 ± 1.7
α_2	-21.4 ± 3.4	62.8 ± 3.5	84.0 ± 4.8
α_4	42.0 ± 4.9	-62.0 ± 4.5	-104.1 ± 6.6
α_6	-19.8 ± 5.8	-181.7 ± 6.4	-161.9 ± 8.6

frequency variations with time, but again the signal-to-noise ratio has been fairly low and the results derived from different observations are inconsistent^{10–12}.

One area where many different observers have been in relatively good agreement is the time variation of the even-index p-mode splitting coefficients. If we expand the p-mode frequencies of a single (nl) multiplet as a sum of Legendre polynomials

$$\nu_{nlm} = \nu_{nl} + \sum_{i=1} \alpha_i(n, l) P_i(m/L) \quad (1)$$

with $L = \sqrt{l(l+1)}$, then the α_i with even i measure a latitude-dependent variation in the effective propagation speed for acoustic waves (owing to, for example, real variations in the speed of sound inside the Sun and changes in the structure of the surface boundary), and the α_i with odd i measure a corresponding east-west propagation asymmetry (the solar rotation). Kuhn¹³ first suggested that the observed $\bar{\alpha}_2$ and $\bar{\alpha}_4$ (here averaging $\alpha_i(n, l)$ over observed n and l) varied systematically with solar cycle, and all the measurements to date, made by several different observers, support this^{10,14,15}.

The data presented here at Big Bear Solar Observatory using our dedicated helioseismology telescope^{16,17}, over a period of approximately four months in the summer of 1986 and again in 1988. The very long observing runs resulted in very accurate measurements of p-mode frequencies, as shown in Fig. 1. The 1986 data set has already been used to measure p-mode amplitudes and linewidths^{4,17}, splittings^{15,18} and accurate mode frequencies¹⁹. Because 1986 was near solar minimum whereas by 1988 the new magnetic cycle had reached considerable amplitude, these data are well suited to search for solar-cycle frequency changes.

Frequency shifts

The change in the mode frequencies, $\Delta\nu_{nl} = \nu_{nl}(1988) - \nu_{nl}(1986)$ depends strongly on mode frequency ν_{nl} and only weakly on l (Fig. 2). the data are fairly well described by $\Delta\nu(\nu, l) \propto M^{-1}(\nu, l)$, where M is the so-called mode ‘mass’, defined as the ratio of the mode energy to the square of the surface velocity, the latter evaluated at a 5,000-Å optical depth of $\tau_{5,000} = 0.05$.

We suggest that the most significant solar-cycle changes in the Sun’s structure, inasmuch as they affect the p-mode frequencies, occur near the surface. Such a hypothesis is qualitatively supported by the observed $\Delta\nu(\nu, l)$, and can be understood by considering the details of the radial structure of the mode eigenfunctions. For a given l , the upper reflection point for lower- ν modes is deeper in the Sun than for higher- ν modes. At fixed frequency, the eigenfunctions are roughly l -independent near the surface, but lower- l modes penetrate more deeply into the solar interior than higher- l modes. Consequently higher- ν , and to a lesser extent higher- l modes, are more sensitive to surface perturbations, which is consistent with the observed $\Delta\nu(\nu, l)$.

If instead the perturbation were to extend over a significant fraction of the solar radius, then from asymptotic theory the (fractional) mode frequency shift would depend mainly on the ratio ν_{nl}/l , which labels different acoustic rays. Because the observed $\Delta\nu(\nu, l)$ is not well described as a function of ν_{nl}/l we conclude that the relevant structure changes occur mainly in a thin layer. The effect of perturbing a thin layer in the propagating regions of the modes was studied by Thompson²⁰, who found an oscillatory frequency dependence in $\Delta\nu$, which we do not observe. Thus the dominant effect on the frequencies is not the direct result of, say, changes in the magnetic field at the base of the convection zone, although the effect of such a hypothetical perturbed layer could conceivably show up in a more careful analysis. A perturbation confined mainly to the evanescent regions of the modes near the centre of the Sun can also be ruled out because the frequency shift does not increase with decreasing l .

Without resorting to a detailed model, it is our view that the p-mode frequencies are responding to changes in the strength of solar magnetic activity near the Sun’s surface. In addition to

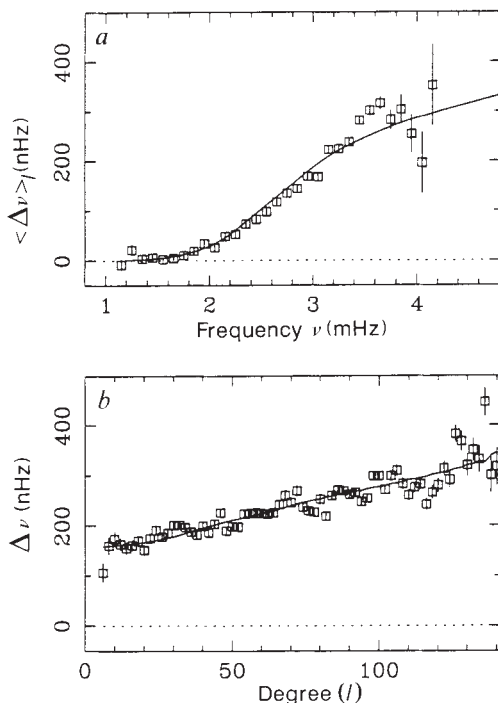


FIG. 2 *a*, p-mode frequency differences between 1988 and 1986, $\Delta\nu = \nu_{88} - \nu_{86}$, as a function of frequency, after averaging over measured modes with degree $5 \leq l \leq 60$. The solid line is the inverse mode mass at $l=20$, $M^{-1}(\nu, l=20)$, scaled to fit the data, using mode masses at $\tau_{5,000} = 0.05$ (P. Kumar, personal communication) based on a solar model by Christensen-Dalsgaard²⁶. *b*, p-mode frequency differences at 3 mHz, as a function of spherical harmonic degree l . The points were obtained by fitting the observed frequency shifts in each l range to $M^{-1}(\nu, l=20)$ and evaluating the fit at 3 mHz. Also plotted is the inverse mass function at 3 mHz, $M^{-1}(\nu=3 \text{ mHz}, l)$, scaled to fit the data.

the direct effect of magnetic fields on the dynamics of oscillatory motion, the fields can modify the thermal structure of the Sun, particularly near the upper boundary, on which the mode frequencies depend.

The surface evanescent layer of a typical observed mode extends to a depth $\approx 1\%$ of the solar radius, so it is likely that the perturbation is not strong much deeper than this. On the other hand, a simple calculation of the effect of a perturbation confined strictly to the photosphere yields an even stronger frequency dependence for $\Delta\nu(\nu, l)$ than is observed. The calculation, based on a Lagrangian formalism^{14,21}, assumed that the vertical extent of the perturbed layer does not exceed one pressure scale height and used mode eigenfunctions appropriate to an isothermal photosphere. The effects of a surface perturbation were calculated, in an approximate way, within this formalism, and the treatment was simplified by considering $l=0$ modes only. For modes with frequencies below the acoustic cutoff, the rough frequency dependence of $\Delta\nu$ was found to be

$$\Delta\nu \propto \frac{\nu^3}{M(\nu)} \quad (2)$$

where $M(\nu)$ is the mass of the $l=0$ modes evaluated at the location of the perturbation.

As the observations are reasonably well fit by a simple $M(\nu)^{-1}$ dependence at low l , we conclude that the observed $\Delta\nu$ cannot originate solely in the photosphere, and therefore the sub-photospheric layers must contribute significantly to the observed shift. It should be noted that the choice, $\tau_{5,000} = 0.05$, at which M was evaluated for comparison with the data is not crucial to this

conclusion, because the function $M(\nu, l)$ at this optical depth adequately represents the frequency dependence the mode mass for photospheric values of τ . P. Goldreich *et al.* (personal communication) have found from detailed modelling that the observed $\Delta\nu(\nu, l)$ can follow from quite reasonable assumptions about how the top of the convection zone is perturbed as the result of solar magnetic activity.

Frequency splittings

The above $\Delta\nu(\nu, l)$ suggest that between 1986 and 1988 there was a change in the structure of near-surface layers of the Sun, a change that affected the propagation of acoustic waves in that region. If the responsible time-dependent perturbation also depends on latitude, as one might expect for a solar-cycle effect, then it should also be observable in the even-index splitting coefficients $\alpha_{2j}(n, l)$ in both the 1986 and 1988 data sets. In particular, if the perturbation can be separated into the product of a time-independent radial function and a time-dependent function of latitude, then the α_{2j} should show the same frequency dependence as the $\Delta\nu$. To investigate this hypothesis, we measured the frequency splittings $\alpha_i(n, l)$ in equation (1) up to $i=6$. Averaging over l for $5 \leq l \leq 60$ we obtain the results shown in Fig. 3 for the 1986 and 1988 data. Clearly all the α_{2j} show a frequency dependence that is consistent with the inverse mass function. We also found that the α_{2j} depend only weakly on l for $5 \leq l \leq 60$, consistent with the measured $\Delta\nu(\nu, l)$.

We have not corrected the data in Fig. 3 for the second-order effects of solar rotation, which are expected to produce a measurable α_2 . The calculated rotational α_2 is small (P. Goode, personal communication), however, smaller even than the observed

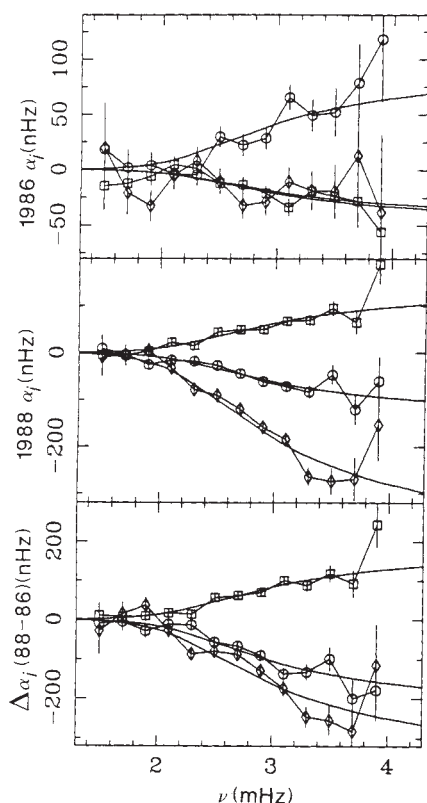


FIG. 3 Plot of the even-index splitting coefficients, α_i , $i=2, 4, 6$, as a function of frequency for 1986 and 1988, averaging over measured multiplets with $5 \leq l \leq 60$. α_2 , α_4 and α_6 are represented by boxes, circles and diamonds, respectively. Lines connect the data points, and fits to $M^{-1}(\nu, l=20)$ are also drawn for each of the α_i . As for the $\Delta\nu$ data in Fig. 2a, the frequency dependence of the α_i is well represented by $M^{-1}(\nu, l=20)$, particularly in 1988.

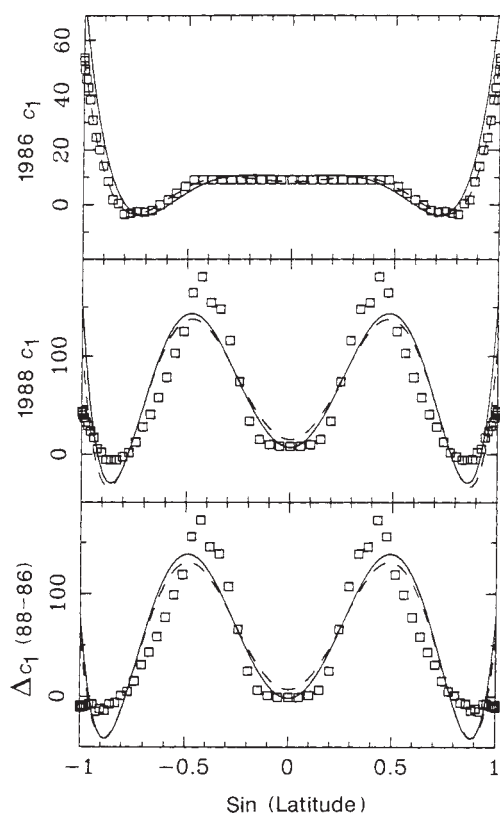


FIG. 4 Latitude inversions of the splitting data, compared with solar-limb brightness measurements. The solid lines are the inversions, the points are the limb measurements, and the dotted lines are fits to the limb measurements using equation (3), keeping only terms up to β_6 . All the plots contain the same arbitrary scale factor.

α_2 in 1986, so little is lost by neglecting this effect. Dziembowski and Goode performed an inversion of the 1986 splittings, and found evidence for a strong magnetic field at the base of the convection zone²². This seems to contradict our conclusion above that the dominant perturbation is near the solar surface, but further work may clarify this.

Assuming that the dominant cause of the measured non-zero α_{2j} is a near-surface perturbation, the data support our hypothesis that the radial structure of solar magnetic activity near the surface is independent of both time and latitude. The support is not irrefutable, however, because in 1986 the signal-to-noise ratio in the α_{2j} measurements is just high enough to discern the $M^{-1}(\nu)$ functional form, and in 1988 the signal is dominated, as we will see below, by solar activity that is concentrated in a fairly narrow latitude band.

Averaging the $\Delta\nu$ and α_{2j} over $5 \leq l \leq 60$, fitting each to $M^{-1}(\nu, l=20)$, and evaluating the fit at $\nu = 3$ mHz, we obtain the fit coefficients given in Table 1.

Latitude inversions

It is straightforward and instructive to invert the α_{2j} and $\Delta\nu$ to obtain a measure of the strength of the perturbation as a function of solar latitude, assuming as above that its radial structure is independent of both time and latitude. Expressing the perturbation as an effective sound-speed perturbation $c_1(r, \theta, t)$, we can then write^{13,23,24}

$$c_1(r, \theta, t) = \sum_j \beta_{2j}(t) f(r) P_{2j}(\cos \theta) \quad (3)$$

where θ is the colatitude, and the observable splitting coefficients are

$$\alpha_{2j}(\nu, l, t) = C(\nu, l) (-1)^j \frac{(2j-1)!!}{(2j)!!} \beta_{2j}(t) \quad (4)$$

where $C(\nu, l)$ is a function determined by the depth dependence $f(r)$ of the perturbation. This result is a good approximation when $j \ll l$, and therefore applies to all but the lowest- l modes studied here. Equation (4) also holds for $j=0$ if we identify $\Delta\alpha_0 = \Delta\nu$, giving $\Delta\nu(\nu, l) = C(\nu, l) \Delta\beta_0$. If we take $C(\nu, l) \propto M^{-1}(\nu, l)$, then we see from Figs 2 and 3 that all the data are consistent with our assumption of a time- and latitude-independent $f(r)$. It remains to be seen whether this consistency persists over the solar cycle, as better data are acquired.

Using the fit coefficients in Table 1, the depth dependence of the perturbation can be absorbed into a single scale factor, allowing an inversion to determine the latitude dependence of the effective perturbation. We expect that such an inversion will show that the perturbation is primarily confined to the active latitudes on the Sun. To investigate this, we have used the limb photometer measurements of Kuhn *et al.*^{13,25}. (Because there

are no 1986 limb data, we interpolated the data from 1985 and 1987 to estimate the 1986 limb brightness.) These data measure the relative effective temperature of the solar limb, and thus serve to outline the active latitudes, although the detailed physical changes near the solar surface which are responsible for the limb observations have yet to be identified.

The results of the inversions are shown in Fig. 4, for 1986 and 1988, as well as for the difference between the 1988 and 1986 data. Because β_0 cannot be determined absolutely, the 1986 and 1988 inversions were arbitrarily displaced to match the limb data. Comparing 1988 and 1986 data, however, we can determine $\Delta\beta_0$ along with the other $\Delta\beta_{2j}$, so the (1988–1986) inversion contains no arbitrary displacement. All the inversions were scaled using a single scale factor.

The fit of the limb temperature data to the splitting inversions is remarkably good. The splittings clearly give the expected result in that the effective sound-speed perturbation is confined mostly to the active latitudes when the activity is strong. The 1986 splittings show a substantial perturbation coming from the polar region, however, which again matches the limb brightness measurements. The presence of the polar perturbation results from the relatively large α_4 seen in 1986. Therefore the source of the perturbation cannot be the very strong atmosphere perturbation in active regions alone, because there are no active regions at the solar poles. Weaker magnetic fields, or large-scale temperature variations, must contribute significantly to the perturbation.

Discussion

These new measurements clearly show that p-mode frequencies change with time, presumably owing to changes in the structure of the Sun during the solar cycle. The measured frequency shifts $\Delta\nu(\nu, l)$ depend strongly on frequency and weakly on l for $5 \leq l \leq 140$, with a functional form that is well described by $\Delta\nu(\nu, l) \propto M^{-1}(\nu, l)$, the latter evaluated at optical depth, $\tau_{5,000} = 0.05$. This frequency dependence indicates that the dominant structural changes during the solar cycle, inasmuch as they affect p-mode frequencies, occur near the solar surface, although we do not yet have a detailed physical model of the perturbation. Further, the even-coefficient p-mode splittings show the same frequency dependence as the $\Delta\nu$, which suggests that the radial structure of the near-surface perturbation is independent of both time and latitude. Inversions of the splittings show that the hypothesized near-surface perturbation is strongest in the active latitudes, but includes a weak polar component at the solar-cycle minimum. These data open up the possibility of inverting $\Delta\nu(\nu, l)$ and $\alpha_{2j}(\nu, l)$ to determine the detailed depth dependence of the perturbation, and hopefully of constructing a model that describes the structure of solar activity as a function of depth. $\square$

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Direct and selective binding of an acidic transcriptional activation domain to the TATA-box factor TFIID

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The potent transactivation domain of the herpes simplex virion protein VP16 was used as a column ligand for affinity chromatography. VP16 binds strongly and highly selectively to the human and yeast TATA box-binding factors. Our results imply that the principal target for acidic activation domains is the TATA-box factor TFIID.

TRANSCRIPTION by eukaryotic RNA polymerase II is regulated by sequence-specific DNA-binding activator proteins¹. These proteins, even when bound at considerable distances from the transcription start site, cause RNA polymerase II to initiate more frequently². Many activator proteins can be genetically dissected into two independent domains; one binds to specific DNA sequences, the other activates transcription³. DNA between the bound activator protein and the transcription initiation site probably loops out, allowing the activator protein to contact RNA polymerase II or a general initiation factor at the promoter⁴. These general initiation factors (TFIIA, B, D, E and F), which are essential for RNA polymerase II to initiate transcription at promoter sites⁵⁻⁸, can be assembled into an initiation complex in a defined order^{9,10}. TFIID is the factor that binds to the TATA box present in most promoters transcribed by RNA polymerase II (refs 7, 10, 11). DNA-binding studies suggest that several transcriptional activators might contact TFIID (refs 11-

14); after TFIID and TFIIA are bound to the promoter, TFIIB, RNA polymerase II and TFIIE/F can be incorporated into the initiation complex¹⁰. TFIIF (ref. 8) or RAP30/74 (refs 8, 15, 16) binds to RNA polymerase II (refs 15, 17) and probably catalyses the transition from a closed to open complex at the promoter¹⁸.

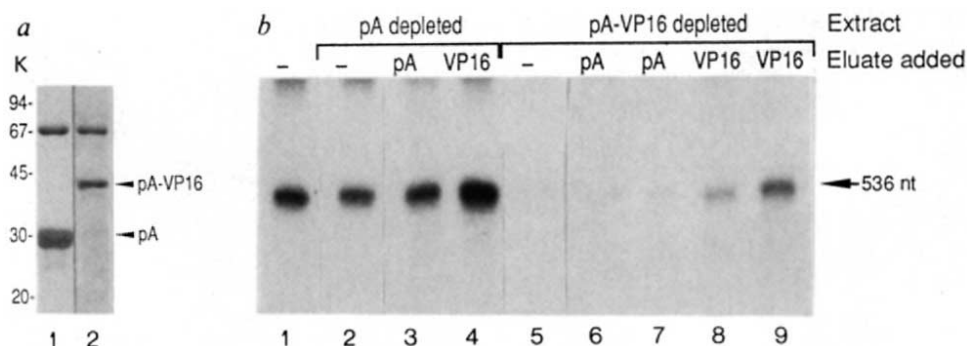
We wished to identify a component of the initiation complex that is contacted directly by a prototypical activation domain. As the herpes simplex virion protein VP16 (refs 19-25) has a particularly potent activation domain of the acidic type²⁶⁻²⁸, we used affinity chromatography to determine whether VP16 could bind directly and selectively to RNA polymerase II or one of its general initiation factors.

VP16 binds an initiation factor

The activation domain of VP16 was expressed in *Escherichia coli* as part of a fusion protein with the IgG-binding domain of *Staphylococcus aureus* protein A (pA), purified by IgG affinity chromatography (Fig. 1a) and coupled to agarose. In experiments with human HeLa cell extracts, affinity columns containing 500 $\mu\text{g ml}^{-1}$ immobilized pA-VP16 selectively bound in salt-elutable form only ~0.05% of the applied protein (data not shown). By quantitating the level of accurately initiated run-off transcription at the adenovirus 2 major late promoter (Ad2MLP), we showed (Fig. 1b) that HeLa nuclear transcription extract flowing through a control pA column was as active as untreated extract (lanes 1, 2), but extract flowing through a

FIG. 1 A general initiation factor in HeLa cell nuclear extracts binds to the activation domain of VP16. **a**, SDS-PAGE of the proteins used to prepare affinity columns: lane 1, protein A (pA); lane 2, protein A-VP16 (pA-VP16). Protein *M*_r standards are indicated (left). **b**, Run-off transcripts initiated at the Ad2MLP in reactions containing untreated HeLa nuclear extract (lane 1) or nuclear extracts that flowed through a control column of pA-agarose (lanes 2-4) or through a column of pA-VP16-agarose (lanes 5-9). Reactions were supplemented with buffer D (ref. 17) (lanes 1, 2, 5) or with the proteins in a whole-cell extract eluted with 0.57 M KCl from the pA column (lanes 3 and 7, 2 μl ; lane 6, 1 μl) or from the pA-VP16 column (lanes 4 and 9, 2 μl ; lane 8, 1 μl). Nucleotides, nt.

METHODS. **a**, DNA encoding the C-terminal 79 amino acids of HSV VP16 (ref. 44) was EcoRI-linked and cloned into the protein A fusion vector pRIT2T (Pharmacia). The *E. coli* strain N4830-1 was transformed with pRIT2T or the pRIT2T-VP16 DNA. Synthesis of pA or pA-VP16 was induced by growth at 42 °C for 90 min and both polypeptides were purified on IgG columns (Pharmacia) following the manufacturer's instructions for low pH elutions. Aliquots of the two preparations were analysed on a 12.5% gel and stained with Coomassie blue. **b**, Protein A and protein A-VP16 preparations were dialysed for 2 h against 10 mM HEPES buffer, pH 7.9, 500 mM NaCl, 0.1 mM EDTA, 0.1 mM dithiothreitol and 10% glycerol, then coupled⁴⁵ to Affigel 10 (Biorad) at a final concentration of 500 μg protein per ml gel. Aliquots of dialysed (400 μl , 5 h at 4 °C against 20 mM HEPES, pH 7.9, 100 mM KCl, 0.2 mM EDTA, 0.2 mM EGTA, 2 mM dithiothreitol, 20%



glycerol) HeLa nuclear extract⁴⁵ were chromatographed at 4 °C on 200- μl columns of pA-agarose or pA-VP16-agarose. After recycling the flow-through fractions through the columns four times, each column was washed with 400 μl buffer and the flow-through and wash fractions were pooled and stored at -70 °C. Dialysed whole-cell extract (8 ml)^{15,17} derived from 2 g HeLa cells were also chromatographed on the same columns in 10 mM HEPES, pH 7.9, 100 mM KCl, 0.2 mM EDTA, 1 mM dithiothreitol and 10% glycerol. The columns were washed with 3 ml buffer and bound proteins were eluted with 0.6 ml buffer containing 0.57 M KCl. The pA-VP16 column eluate contained only 1% of the applied RNA polymerase II and 0.1% of the applied RAP30 (data not shown). *In vitro* run-off transcription assays using Ad2MLP DNA were performed and analysed on 5% polyacrylamide-urea gels as previously described^{16,17}. Reactions (20 μl) contained 2 μl untreated nuclear extract or 4 μl column flow-through fractions, 400 ng pMLP DNA¹⁶ digested with *Sma*I and 1 or 2 μl of the 0.57 M KCl column eluates, as indicated above.

pA-VP16 column was virtually inactive (lane 5). Only salt eluate from the pA-VP16 column (Fig. 1b, lanes 8, 9), and not eluate from the pA column (lanes 6, 7), could restore activity to extract that had been inactivated by passage through the pA-VP16 column. Therefore, we concluded that the pA-VP16 column must be binding RNA polymerase II and/or at least one of the general initiation factors. The eluate from the pA-VP16 column also stimulated transcription by untreated nuclear extract (Fig. 2, lanes a-c) and by extract that had been passed over a control pA column (Fig. 1b, lane 4). As TFIID is the limiting factor for initiation on the Ad2MLP in a HeLa nuclear extract²⁹, we thought that the transcription factor that bound to the pA-VP16 column might be the TATA-box factor TFIID.

VP16 binds human TFIID

To test whether TFIID bound to our pA-VP16 column we initially used HeLa nuclear extract in which TFIID had been selectively inactivated by heating at 47 °C (ref. 29). Such an inactive heated extract (Fig. 2, lane d) was reconstituted with eluate from a pA-VP16 column (lane f), but not with eluate from a pA column (lane e). Routinely, 50–70% of the TFIID activity in HeLa extract was recovered in the pA-VP16 column eluate. Moreover, most of the activity in the pA-VP16 column eluate also behaved chromatographically like TFIID. The main peak of activity eluted from a DE52 column at 0.17 M KCl (Fig. 3), as does human TFIID (ref. 29).

An independent assay for TFIID activity is based on its requirement for template commitment at TATA box-containing promoters⁷. In our template commitment experiment (Fig. 4) the two Ad2MLP templates produced run-off transcripts of different sizes because they were digested with different restriction enzymes (Fig. 4, lane a). When template 1 or 2 was preincubated with the HeLa nuclear extract before the other template was added, no transcript was produced from the second template (lanes b, e). The transcription of the second template, however, was restored if the eluate from the pA-VP16 column (lanes d, g), but not the eluate from the pA column (lanes c, f), was added to the reaction together with the second template. Therefore, by this additional criterion, the eluate from the pA-VP16 column contained human TFIID.

VP16 binds yeast TFIID

To establish whether VP16 binds directly to TFIID or to a complex that included other transcription factors, we made use of the fact that the gene for *Saccharomyces cerevisiae* TFIID

has been cloned^{30–34} and expressed in *E. coli*³¹. The yeast TFIID polypeptide of relative molecular mass 27,000 (M_r 27K) was a minor protein (~0.05% of total protein) and could not be readily identified on SDS-polyacrylamide gels when an extract from bacteria containing the TFIID-encoding plasmid pASY2D (ref. 31) was compared with extract from bacteria containing pASD2Y, in which the TFIID coding sequence was in the reverse orientation (Fig. 5a, lanes 1, 2). The 27K TFIID polypeptide that was present only in the pASY2D extract bound to the pA-VP16 column (Fig. 5a, lane 6) and not to the control pA column (lane 4). Therefore, the binding of yeast TFIID to VP16 did not require any additional eukaryotic proteins.

Two unidentified *E. coli* proteins, which also bound selectively to the pA-VP16 columns (M_r ~34K and 18K, Fig. 5a, lanes 5, 6), were eliminated when TFIID was purified to virtual homogeneity on a larger scale. This purified yeast TFIID, when re-chromatographed, bound to a pA-VP16 column, but not to a pA column (Fig. 5, lanes 3, 2). Therefore, the interaction between yeast TFIID and VP16 does not require any other polypeptide. As the human TFIID in a HeLa extract bound to VP16 under the same chromatographic conditions, our data imply that human TFIID also binds directly to VP16.

Yeast TFIID can functionally replace human TFIID *in vitro*^{35,36}. Yeast TFIID purified by VP16 chromatography reconstituted transcription from the Ad2MLP in a heat-treated HeLa nuclear extract (Fig. 6, lanes a–d) and in an extract that had been partially inactivated by passage through a pA-VP16 column (lanes g, h). TFIID seems, therefore, to be the only general initiation factor that is efficiently depleted from a HeLa extract by VP16 affinity chromatography. By this criterion, TFIID is either the only general initiation factor that binds to the activation domain of VP16, or the one that binds most tightly.

Discussion

We have shown that the acidic activation domain of herpes simplex virus VP16 binds to the human and yeast TATA-box factors. In both cases the binding was so highly selective that

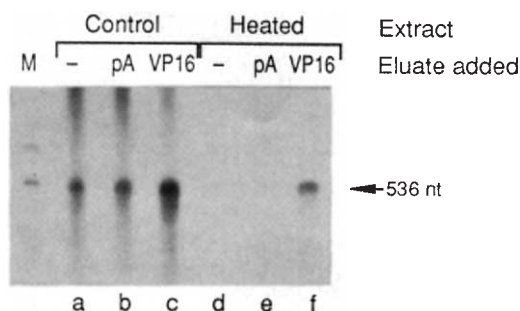


FIG. 2 The HeLa cell initiation factor that binds VP16 restores activity to a heat-inactivated HeLa nuclear extract. *In vitro* transcription reactions with control (lanes a–c) or heat-inactivated (6 min at 47 °C; lanes d–f) HeLa nuclear extracts were supplemented with 5 μ l buffer D (ref. 17; lanes a, d) or 5 μ l of the dialysed 0.57 M KCl eluates from the pA (lanes b, e) or pA-VP16 (lanes c, f) columns that had been loaded with HeLa whole cell extract (see Fig. 1). The marker lane (M) contains end-labelled *Hpa*II-digested pBR322 DNA. Nucleotides, nt.

METHODS. *In vitro* transcription reactions in 20 μ l contained 600 ng *Sma*I-digested pMLP DNA and 3.5 μ l HeLa nuclear extract. The 0.57 M KCl eluates from the pA and pA-VP16 columns had been dialysed against buffer D for 3.5 h.

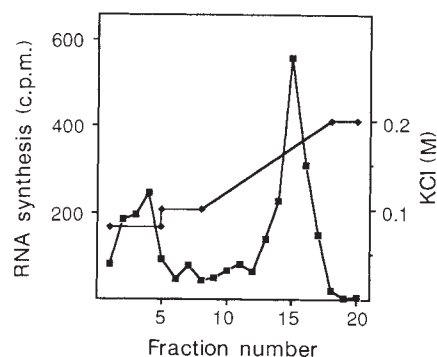


FIG. 3 Chromatography of the VP16-bound transcription factor on DEAE-cellulose. Components of the HeLa whole-cell extract that bound to, and were eluted from, a VP16 column were fractionated further by DEAE-cellulose chromatography. Aliquots of each fraction were assayed in the absence of GTP for their ability to stimulate transcription of the cassette template lacking G residues, pMLC₂AT (ref. 46) by heat-inactivated HeLa nuclear extract lacking TFIID activity. RNA synthesis, ■; KCl concentration, ◆. METHODS. A 300- μ l aliquot (of 1.5 ml) of the 0.57 M KCl eluate from a 1.5-ml protein A-VP16 column over which 75 ml HeLa whole cell extract had been chromatographed (see Fig. 1) was dialysed against DE52 buffer²⁹ and then loaded over 1.5 h onto a 50 μ l DE52 (Whatman) column. After a 200 μ l buffer wash, the column was eluted by successive 25- μ l steps, each having a 10-mM increment in KCl concentration from 0.1–0.2 M. A 5- μ l aliquot of each 25- μ l fraction was assayed in 20- μ l reactions containing 12 mM HEPES buffer, pH 7.9, 57–82 mM KCl, 3 mM EGTA, 12 mM MgCl₂, 12% glycerol, 20 μ g ml⁻¹ pMLC₂AT DNA⁴⁶, 600 μ M ATP and CTP, 30 μ M [³H]UTP (1.7 Ci mmol⁻¹) and 2.5 μ l heat-inactivated (47 °C, 6 min) HeLa nuclear extract. Reactions were incubated for 60 min at 30 °C and total RNA synthesis determined¹⁵.

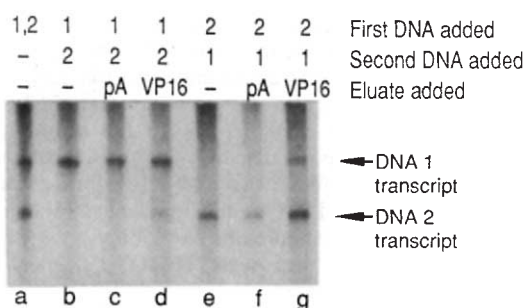


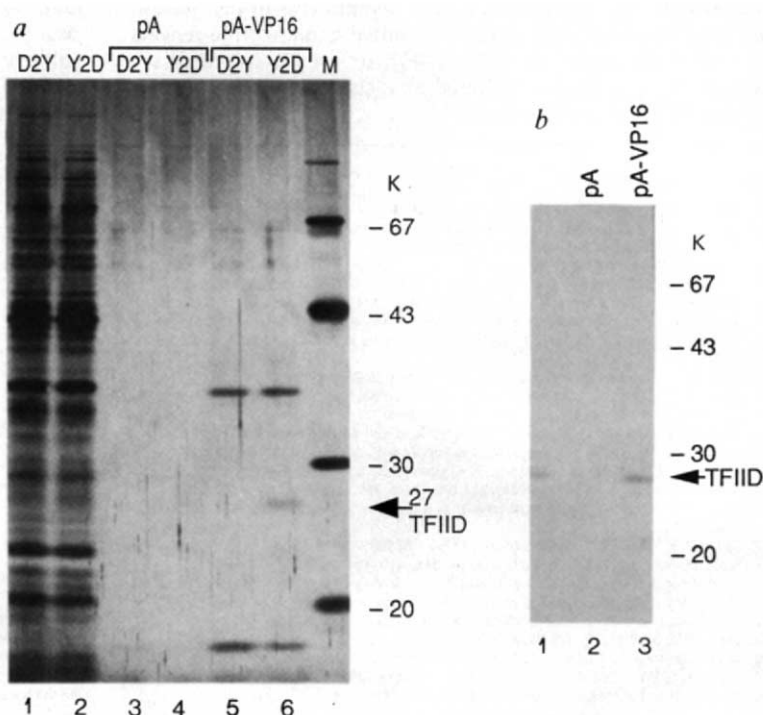
FIG. 4 The HeLa cell initiation factor that binds VP16 provides TFIID activity in a template-commitment experiment. *In vitro* transcription reactions were performed in two steps. HeLa nuclear extract was pre-incubated with DNA

template 1 or 2 (*Sma*I- or *Hinc*II-digested pMLP DNA, leading to 536- or 405-nucleotide run-off transcripts, respectively) as indicated. Transcription was then initiated by addition of the second DNA and nucleotides. The second DNA had been pre-incubated either with buffer D (ref. 17) (lanes a, b, e) or with the dialysed 0.57 M KCl eluates from pA (lanes c, f) or pA-VP16 (lanes d, g) columns.

METHODS. The first incubation (9 μ l; 30 min at 30 $^{\circ}$ C) contained 2.2 μ l HeLa nuclear extract, 60 mM KCl, 12 mM $MgCl_2$, 12 mM HEPES buffer, pH 7.9, 3 mM EGTA, 0.2 mM dithiothreitol, 0.2 mM phenylmethyl sulphonyl fluoride, 12% glycerol and 500 ng DNA 1 or 2, as indicated. To this was then added 9 μ l pre-incubated (30 min, 30 $^{\circ}$ C) mixture containing, in the same buffer, 500 ng second DNA template and 4.5 μ l either buffer D or the dialysed 0.57 M KCl eluates from the pA or pA-VP16 columns. Transcription was immediately initiated by a further 2- μ l addition of 1.8 U RNA Guard (Pharmacia) and nucleotides ATP, CTP and UTP (6.7 mM each) and 0.3 mM [α - 32 P]GTP (8 Ci mmol $^{-1}$). The resulting mixture (20 μ l) was incubated for 17 min at 30 $^{\circ}$ C and the run-off transcripts then processed (see Fig. 1).

FIG. 5 Yeast TFIID made in *E. coli* binds directly to VP16. *a*, A 10% SDS-polyacrylamide gel was used to analyse polypeptides present in extracts of *E. coli* cells containing a control plasmid (pASD2Y, lane 1) or a yeast TFIID expression plasmid (pASY2D), lane 2), as well as the polypeptides in these extracts that bound to pA (lanes 3, 4) or pA-VP16 (lanes 5, 6) columns. *M_r* size markers on the right (*M*). *b*, Re-chromatography of affinity-purified yeast TFIID on control pA and pA-VP16 columns. An aliquot of the TFIID preparation applied to the column (lane 1) and the protein bound to, and then eluted with 0.5 M NaCl from, a pA (lane 2) or a pA-VP16 (lane 3) column, were analysed on a 12.5% SDS-polyacrylamide gel.

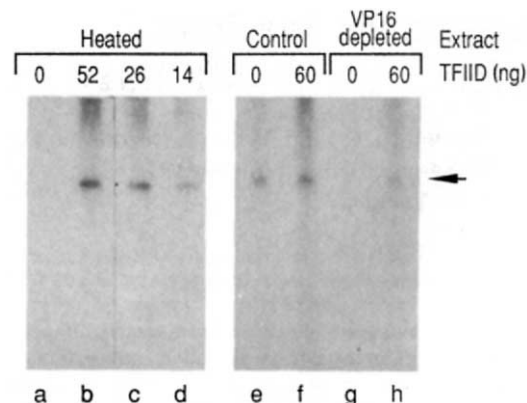
METHODS. *a*, *E. coli* cells containing pASD2Y or pASY2D (ref. 31) were grown in L-broth (10 g Bacto-tryptone, 5 g yeast extract, 10 g NaCl per litre) ampicillin (20 μ g ml $^{-1}$) medium at 30 $^{\circ}$ C to an absorbance at 600 nm of 0.7. An equal volume of 65 $^{\circ}$ C medium was added and growth continued for 20 min at 42 $^{\circ}$ C. The cultures were then chilled with crushed ice, the cells collected by centrifugation and resuspended in 20 ml g $^{-1}$ cold 25 mM Tris-HCl buffer, pH 8.0, 10 mM EDTA, 50 mM sucrose, 1 mM dithiothreitol, 1 mM phenylmethyl sulphonyl fluoride. Extracts were prepared by lysozyme (1 mg ml $^{-1}$) treatment (30 min, 0 $^{\circ}$ C), addition of KCl to 0.5 M, centrifugation (20 min, 150,000g) and dialysis of the supernatants (3 h, 4 $^{\circ}$ C) against affinity-column buffer¹⁵. Aliquots of these extracts (200 μ l) were loaded on 20- μ l pA or pA-VP16 columns, the columns washed with 200 μ l buffer and then eluted with 60 μ l buffer containing 0.5 M NaCl. The initial extracts (1 μ l) or 15 μ l of the 0.5 M NaCl column eluates were analysed. The gel was silver-stained⁴⁷. *b*, Purified yeast TFIID was prepared from a 10 culture of *E. coli* cells containing pASY2D by a further scale-up of the method described in the legend to Fig. 6 (final yield 1.5 mg; details available on request). The purified TFIID (3 μ g) was loaded on 20- μ l pA or pA-VP16 columns. The columns were washed with



200 μ l buffer and then eluted with 60 μ l buffer containing 0.5 M NaCl. For gel electrophoresis, 1.5 μ g purified TFIID and all of the 0.5 M NaCl eluates were analysed. The gel was stained with Coomassie blue.

FIG. 6 Yeast TFIID purified by VP16 affinity chromatography restores activity to HeLa nuclear extracts inactivated by heating, or depleted by pA-VP16 chromatography. *In vitro* transcription reactions contained HeLa nuclear extract that was heated (lanes a-d), or passed through a control pA (lanes e, f) or a pA-VP16 (lanes g, h) column. Reactions were supplemented with affinity-purified yeast TFIID as indicated.

METHODS. *In vitro* transcription reactions (20 μ l; 60 min at 30 $^{\circ}$ C) contained 10 mM HEPES buffer, pH 7.9, 50 mM KCl, 8 mM $MgCl_2$, 3 mM EGTA, 0.2 mM dithiothreitol, 0.2 mM phenylmethyl sulphonyl fluoride, 10% glycerol, 0.04% Nonidet P-40, 600 μ M ATP, CTP and UTP, 30 μ M [α - 32 P]GTP (8 Ci mmol $^{-1}$), 1.8 U RNA Guard, and either 500 ng pMLP DNA digested with *Sma*I (lanes a-d; 536-nucleotide run-off transcript) or *Hinc*II (lanes e-h; 405-nucleotide run-off transcript), either 3 μ l heated (8.5 min, 47 $^{\circ}$ C) HeLa nuclear extract or 8 μ l HeLa nuclear extract that had been passed over a pA or pA-VP16 column (see Fig. 1) and the indicated amount of affinity-purified yeast TFIID. Affinity-purified yeast TFIID was prepared from a 4-l culture of *E. coli* cells containing pASY2D (ref. 31). The cells were grown and lysed as described in Fig. 5. After centrifugation (90 min, 150,000g), the supernatants were dialysed (6 h, 4 $^{\circ}$ C) against affinity-column buffer¹⁵, clarified by centrifugation and passed (60 ml h $^{-1}$) through a 2.5 $\times$ 12-cm DE52 (Whatman) column equilibrated with the same buffer. The pooled flow-through was adjusted to 20% glycerol and loaded (5 ml h $^{-1}$) on a 1.0-ml pA-VP16 column (see Fig. 1). After a 13-ml wash with buffer containing 20% glycerol, the column



was eluted with buffer containing 20% glycerol and 0.5 M NaCl. The pooled eluate was dialysed (2 h, 4 $^{\circ}$ C) against 10 mM HEPES buffer, pH 7.9, 100 mM KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethyl sulphonyl fluoride and 50% glycerol and stored at -70 $^{\circ}$ C. The final yield was 1 ml containing 200 μ g TFIID, estimated by comparison with protein standards after SDS-PAGE and Coomassie-blue staining.

1,000-fold purification was easily achieved by chromatography of cell extracts on a pA-VP16 column. This has been very useful for the purification of yeast TFIID from bacterial extracts and in the isolation and identification of human TFIID (data not shown). Our results imply that 'squenching' (refs 19, 24, 37) may be caused by sequestration of TFIID, and substantiate the conclusions drawn from previous experiments in which activator proteins affected the binding to DNA of partially purified human TFIID (refs 11-14). We can also conclude that the binding of an acidic activator to TFIID does not require any other protein and that neither the activator protein nor TFIID need to be bound to DNA for the interaction to occur. There is substantial evidence, however, that transcriptional activators make several cooperative contacts with the general transcription apparatus³⁸⁻⁴⁰. It is therefore possible that acidic activators bind to two or more sites on the TFIID polypeptide, make additional contacts of lower affinity with other components of the general initiation complex such as the C-terminal heptapeptide repeats of RNA polymerase II (refs 41-43), or use an intermediary protein to make further indirect contacts with the RNA polymerase II initiation complex.

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Although we have demonstrated direct and extremely selective binding of TFIID to VP16, we have not addressed the biological significance of this interaction. VP16 mutants with compromised transcription function *in vivo* are now being studied. Single amino-acid changes (for example, Phe to Pro at amino acid 442) that do not alter the net negative charge of the activation domain, completely eliminate the transactivation function of VP16 *in vivo* (D. Cress and S. Triezenberg, manuscript in preparation). We have found that columns containing pA-VP16 with such alterations fail to bind yeast TFIID. This correlation between transcriptional activity *in vivo* and TFIID-VP16 interaction *in vitro* (C.J.I., M. Shales, D. Cress, S. Triezenberg and J.G., manuscript in preparation) implies that the TFIID-VP16 interaction detected by affinity chromatography is important for activation of transcription.

The interaction of VP16 with TFIID must be at least partly ionic because it is sensitive to salt. Other activator proteins have activation domains that are not highly acidic¹ and they are unlikely to bind to the same part of TFIID, if indeed they bind to TFIID at all. They could affect different steps in the complicated pathway¹⁰ leading to initiation of transcription. □

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LETTERS TO NATURE

Rapid variability of the iron fluorescence line from the Seyfert 1 galaxy NGC6814

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LUMINOUS broad-band emission from active galactic nuclei (AGN) is thought to be due to accretion onto a massive black hole. Because X-ray emission shows variability on a faster time-scale than any other wavelength band that has been observed it is assumed to be produced very close to the central energy source. Observations of AGN with the 4,000-cm² Large Area Counters (LACs) aboard the Ginga satellite have obtained the highest quality data on X-ray variability to date, allowing examination of detailed spectra up to 20 keV in energy on relatively short time-

scales¹. We have observed NGC6814, one of the faster² X-ray-variable AGN ($\delta t < 200$ s), with Ginga to obtain data on the variability of the iron fluorescence line, and thus to constrain the distribution of cold gas from which the line originates. A positive correlation measured between the variability of the iron-line flux and the continuum emission suggests an upper limit of 10^{13} cm for the size of the iron reprocessing region.

In Fig. 1 we show the X-ray light curve of NGC6814 in the ~2-20 keV waveband from the first day of observation (observations were made on 28-30 April 1989). Using high-resolution (16 s) light curves (Fig. 2) the shortest time for the source to change by a factor of two was found to be ~50 s, confirming the result of Tennant *et al.*². To study the spectral change as a function of the total flux, data were sorted into four intensity groups. Energy spectra of two of these are shown in Fig. 3. The spectra at energies greater than 4 keV are well fitted by a single power-law model, with absorption by neutral gas, and an emission line near 6.4 keV (detailed spectral fits and temporal analysis will be treated in a separate paper). The best-fit parameters are summarized in Table 1. The photon index, Γ , is nearly constant at 1.42, whereas the flux varies. The energy of the emission line is 6.37 ± 0.09 keV, consistent with the iron fluores-

TABLE 1 Best-fit parameters with a single power-law model

	<i>a</i>	<i>b</i>	<i>c</i>	<i>d</i>
Flux*	4.1 ± 1.0	6.6 ± 1.0	8.6 ± 1.1	11.3 ± 1.1
Photon index Γ	1.45 ± 0.11	1.39 ± 0.06	1.40 ± 0.05	1.44 ± 0.04
Iron-line flux (10^{-5} count s $^{-1}$ cm $^{-2}$)	3.5 ± 3.5	12.8 ± 3.5	19.0 ± 3.8	25.8 ± 4.0
Line energy (keV)	6.37 ± 0.59	6.34 ± 0.16	6.38 ± 0.12	6.39 ± 0.09
Equivalent width (eV)	130 ± 130	300 ± 80	350 ± 70	360 ± 55
Log N_H (cm $^{-2}$)	22.92 ± 0.08	22.77 ± 0.06	22.70 ± 0.05	22.66 ± 0.04
Reduced χ^2	1.11	0.84	1.45	1.32

Error ranges represent the 90% confidence level.

* Flux is in units of 10^{-11} erg s $^{-1}$ cm $^{-2}$ in the 2.3–20.9 keV band.

cence line at 6.4 keV and the redshift of the galaxy ($z = 0.0053$). The discovery that iron fluorescence lines are a common feature of the X-ray spectra of Seyfert 1 galaxies^{3,4} implies that 'cold' (low ionization) gas has a large covering fraction around the central source but the physical site of this material has not been determined^{5–7}. We note that both the time-variability behaviour as well as the detailed spectral parameters are quite different from those seen in the EXOSAT⁸ observations. In particular, the column density seen by Ginga is considerably larger than that seen in four of the five EXOSAT observations and the total range of source intensity (more than a factor of five over the entire observation) seen by Ginga is a factor of two larger (the average source intensity, however, was similar). The spectral index determined from the Ginga data is consistent with the EXOSAT observations.

The most striking result of our spectral fits is the correlated change of the iron-line flux with the continuum flux. The equivalent width of the line is nearly constant at ~ 300 eV, independent of the intensity, except for the faintest case (Table 1, column *a*), where only an upper limit could be placed on the line strength.

As the total X-ray flux changes on a timescale of 50 s, energy spectra were required with the minimum possible integration time to investigate the spectral changes as the source flux varied. The shortest accumulation time in which it was possible to detect the iron line was 256 s, and so 108 spectra were sampled across the observation and were fitted with the above model. The resultant iron-line light curve closely follows that of the total X-ray flux. Figure 1 shows a one-day section of the iron-line light curve (Fig. 1*b*) compared with that of the 3–20 keV flux (Fig. 1*a*). To examine the time delay, a cross-correlation analysis was carried out. No asymmetry or lag was found, indicating that the iron-line emission is delayed by less than 256 s, and thus the bulk of the gas emitting the iron K-line lies no further than ~ 300 light s (10^{13} cm) from the central source.

The very large amplitude of the continuum variability on the

timescale of ~ 50 s indicates that the emission region is smaller than 50 light s, or $\sim 10^{12}$ cm. Theoretical arguments suggest that the continuum cannot arise closer than $5 R_g$ ($R_g = (2GM/c^2)$, where G is the gravitational constant) to the central source) and thus $R_g < 10$ light s and $M \leq 10^6 M_\odot$. Close to the central object, any cold gas is expected to have large bulk velocity v_{gas} due to rotation or free fall, and this would substantially Doppler-broaden any line originating in this region⁹. Our data shows that the iron-line width $\delta E < 1$ keV, and thus $\delta E/E \approx v_{\text{gas}}/c \approx (R_g/2R_{\text{Fe}})^{1/2} \leq 1/6.4$, or $R_{\text{Fe}} > 20 R_g \sin \theta$, where θ is the inclination of the putative disk of radius R_{Fe} responsible for the iron-line radiation⁸. If the gas has an ionization state lower than Fe XVII (consistent with that seen in other Seyfert galaxies), our limit on the energy of the centroid of the line implies that the gravitational redshift z_g is less than 0.015. As $(1+z_g) \approx (1-3R_g/2R_{\text{Fe}})^{-1/2} \times f(\theta, M)$ (where $f(\theta, M)$ is a function of θ and central source mass M)¹⁰, we derive (for simple geometries) $R_{\text{Fe}}/R_g \geq 25$. Setting $R_{\text{Fe}}/R_g \approx 25$, and $R_{\text{Fe}} \leq 10^{13}$ cm from the absence of a lag between the line and continuum, implies a central mass $< 1.4 \times 10^6 M_\odot$, consistent with that derived from continuum-variability arguments.

The measured equivalent width of the iron line (~ 300 eV) and its luminosity ($\sim 10^{41}$ erg s $^{-1}$, $\sim 20 \times L_{\text{H}\beta}$ (ref. 11)) cannot arise from fluorescence of cold material with roughly solar abundance¹², and a column density consistent with that derived from the observed spectra, $N_H \approx 5 \times 10^{22}$ cm $^{-2}$ (assuming that the gas is not strongly ionized). To create this strong an iron line by fluorescence requires a region which has a large column density, $N_H > 5 \times 10^{23}$ cm $^{-2}$ and large covering factor, $\Omega/4\pi > 1/2$, values that may be consistent with a very thick accretion disk.

The total infrared, visible and ultraviolet luminosity^{11,13,14} of NGC6814 is somewhat uncertain because of the relative faintness of the nucleus. Values lie in the range $2 \times 10^{41} \leq L_{\text{IR-UV}} \leq 2.5 \times 10^{43}$ erg s $^{-1}$ and these are roughly consistent with the X-ray luminosity of $L_{2-20 \text{ keV}} \approx 7 \times 10^{42}$ erg s $^{-1}$ and the mean ratio for Seyfert 1 galaxies¹³ of $L_{\text{bol}}/L_{2-20 \text{ keV}} \approx 10$. Our upper limit on the central mass combined with $L_{\text{bol}} \geq 8 \times 10^{42}$ implies an Eddington ratio, $L_{\text{bol}}/L_{\text{Eddington}}$, of ≥ 0.05 . If we use the mean $L_{\text{bol}}/L_{2-20 \text{ keV}}$ value then $L_{\text{bol}}/L_{\text{Eddington}} \geq 0.5$. Alternatively the extrapolated hard X-ray luminosity in the 2–500 keV band alone gives $L_{\text{bol}}/L_{\text{Eddington}} \geq 0.4$. These large values of $L_{\text{bol}}/L_{\text{Eddington}}$ are also consistent with a thick disk.

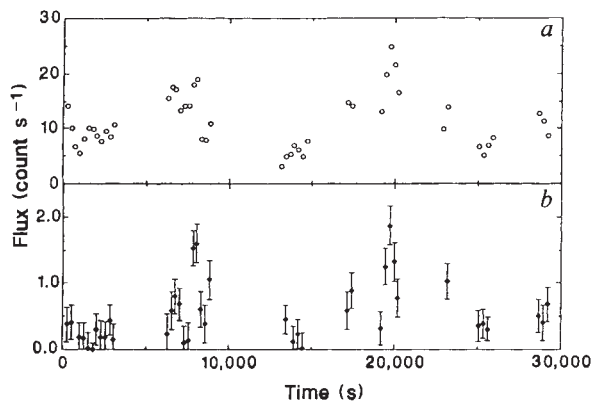


FIG. 1 *a*, Light curve of the continuum X-rays (3.4–20.9 keV) with time bins of 256 s. The error bars of the points are about the size of plotted points (~ 0.5 count s $^{-1}$). *b*, Light curve of the iron-line flux in 256-s bins. The typical 1σ error bar for the line flux is ~ 0.25 count s $^{-1}$ ($\sim 6 \times 10^{-5}$ photon cm $^{-2}$ s $^{-1}$).

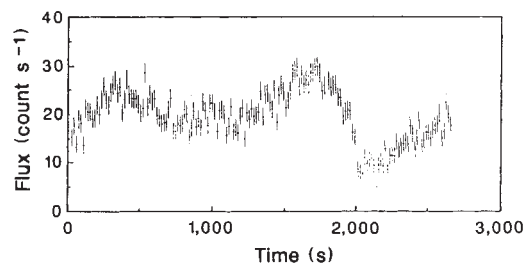


FIG. 2 X-ray light curve in the 2.3–20.9 keV band with 16-s bins. Effective counter area of 4,000 cm 2 .

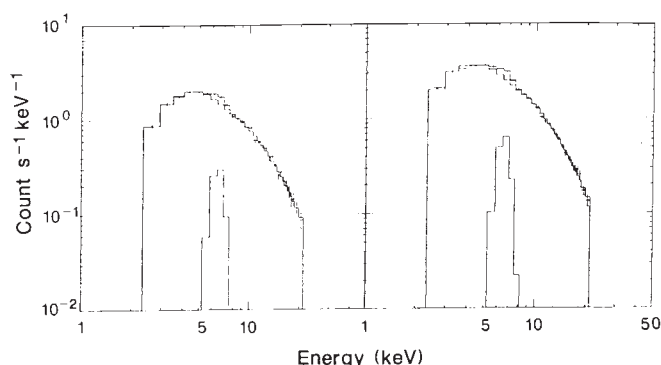


FIG. 3 Left, pulse-height X-ray energy spectra in the faint case (Table 1, column b) and, right, in the bright case (Table 1, column d). The inner histograms represent the best fit to the modelled iron line, and the outer histogram is the best-fit continuum model.

These data provide direct evidence for the existence of cold material, perhaps the accretion flow, close to the central engine of an active nucleus. Our estimates of the mass of the central object, $\sim 10^6 M_{\odot}$, derived from upper limits on the gravitational redshift, the lack of Doppler broadening, and the X-ray-variability timescale, are consistent with one another, and provide evidence that NGC6814 is operating near the Eddington limit. $\square$

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Low-temperature ferromagnetism in $\text{La}_4\text{Ba}_2\text{Cu}_2\text{O}_{10}$

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THERE have been a few reports of ferromagnetic behaviour in the high-transition-temperature (high- T_c) copper oxide superconductors. For example, Na-doped La_2CuO_4 annealed in an inert gas, which is superconducting below 30 K, exhibits a ferromagnetic-like transition near 10 K (ref. 1), although the origin of this behaviour is not clear. In early studies we found that impure samples of $\text{LaBa}_2\text{Cu}_3\text{O}_7$ prepared under a reduced oxygen atmosphere showed evidence of ferromagnetic-like behaviour near 5 K. We have now identified the origin of the ferromagnetic behaviour as being due to the presence of an impurity phase, $\text{La}_4\text{Ba}_2\text{Cu}_2\text{O}_{10}$

(the 422 phase). This phase is an insulator and is homologous to the 'green' phase (Y_2BaCuO_5) found as an impurity in the Y–Ba–Cu–O system. The 422 phase contains square-planar CuO_4 units as do the high- T_c copper oxide superconductors, but in this compound each of these units lies perpendicular to those around it. Magnetization measurements indicate an effective magnetic moment of $1.66 \mu_B$ per copper ion, with a saturation magnetic moment of $0.95 \mu_B$ per Cu. These results suggest that the compound is a localized ferromagnet in which the copper ions are magnetically ordered. We suggest that the ferromagnetic coupling may originate in the orthogonality of the CuO_4 units. There may be analogies with K_2CuF_4 , which is ferromagnetic below 6.5 K (ref. 2) as a result of two-dimensional ordering of the d hole in Cu^{2+} arising from the Jahn–Teller interaction³. The 422 phase could provide flux-pinning centres in bulk superconducting $\text{LaBa}_2\text{Cu}_3\text{O}_{7-y}$.

The 422 phase was first identified by Michel *et al.*⁴ in their studies of the $\text{La}_2\text{CuO}_4 + \text{BaO}$ system. It has also been obtained by decomposing $\text{LaBa}_2\text{Cu}_3\text{O}_{7-y}$ prepared under a reducing atmosphere⁵. It is an insulator, dark brown in colour and with a structure⁴ different from the Y–Ba–Cu–O green phase despite the homology. We prepared 422 by solid-state reaction of the appropriate mixture of high-purity La_2O_3 , BaO and CuO powders. The La_2O_3 powder was preheated at 1,000 °C for 10 h in air because La_2O_3 is very hygroscopic. The mixed powders were calcined at 900 °C for 10 h in air. They were ground and pressed into disks (15-mm diameter, 1-mm thick), and then sintered at 1,000 °C for 10 h in air. The sintered sample was dark brown. The X-ray fluorescence analysis indicates that magnetic impurities other than copper constituted <100 p.p.m. in all samples. Electron-probe microanalysis (EPMA) showed that our samples were essentially in the 422 phase.

The temperature dependence of the d.c. magnetic susceptibility χ and the inverse susceptibility χ^{-1} of 422 were measured in a SQUID (superconducting quantum interference device), using a 50-Oe external magnetic field. The curves obtained (Fig. 1) in both zero-field-cooled and field-cooled runs were almost the same. A Curie–Weiss plot of the data indicates ferromagnetic interaction at 5.2 K. Below 5.2 K, the susceptibility increases abruptly. The Curie constant of 422 corresponds to an effective magnetic moment of $1.66 \mu_B$ per Cu. If this value is assigned to every copper atom, the value of the effective moment is almost the same as the theoretical value for the case in which the orbital angular momentum is completely quenched ($\sqrt{3}$).

The magnetization curves near the magnetic transition temperature are shown in Fig. 2a. Below 5.2 K the magnetization at zero field appears and as the external magnetic field is increased, the magnetization tends towards the saturation value. When the magnetic field sweep width is decreased to 8 mT, the magnetization hysteresis is observed (Fig. 2b). The saturation magnetic moment μ_s is $\sim 0.95 \mu_B$ per Cu. The effective and saturated magnetic moments indicate that almost all Cu^{2+} ions with spin- $\frac{1}{2}$ participate in the localized type of ferromagnetism in 422.

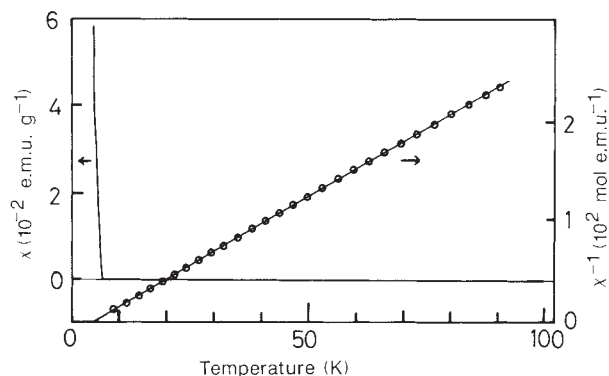


FIG. 1 Temperature-dependent susceptibility χ and its inverse χ^{-1} of 422 cooled in a field of 50 Oe.

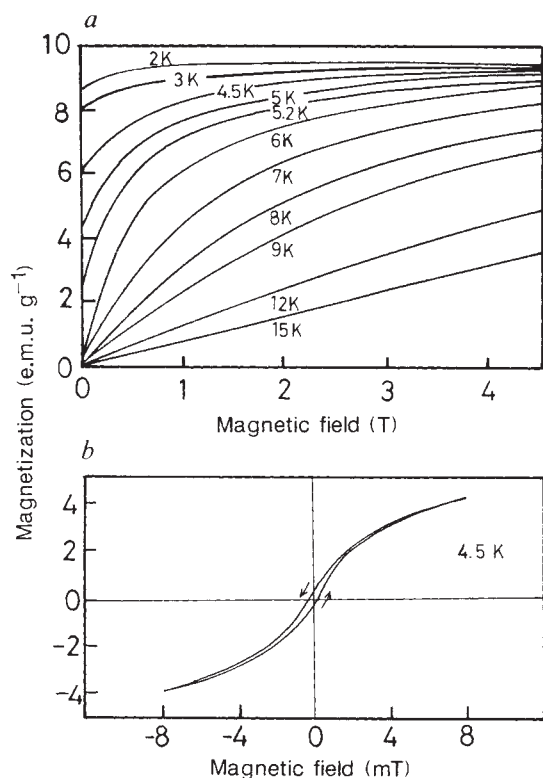


FIG. 2 The magnetization hysteresis curves over 5 T (a) and 8 mT (b) for 422.

To confirm the ferromagnetic transition at ~ 5 K, we investigated the low-temperature specific heat in the range 1.5–6 K. As shown in Fig. 3, there is a large anomaly which suggests a magnetic ordering at ~ 5.2 K. A rough estimate of the magnetic entropy below 5.2 K is $3.6 \text{ J K}^{-1} \text{ mol}^{-1}$, which corresponds to 63% of the theoretical total magnetic entropy. In general, the specific heat is consistent with that expected on the basis of the molecular field approximation for a spin- $\frac{1}{2}$ three-dimensional ferromagnet.

To investigate further the mechanism of the ferromagnetism, we studied the crystal structure of 422 by X-ray powder diffraction and by neutron powder diffraction (using a time-of-flight diffractometer and the pulsed neutron spallation source in the National Laboratory for High-Energy Physics⁶). This compound is tetragonal with space group $P4/mbm$ and lattice parameters $a = 6.8447(9)$ and $c = 5.8637(8)$ Å. According to preliminary neutron diffraction data (N. Ogawa, F.M., H.M., I.H., S.T., T. Mochiku, H. Asano and F. Izumi, unpublished data), LaO_8 and BaO_{10} polyhedra occupy the edge and face positions and the square-planar CuO_4 groups are isolated and are perpendicular to one another (Fig. 4). All the copper sites in 422 are in the

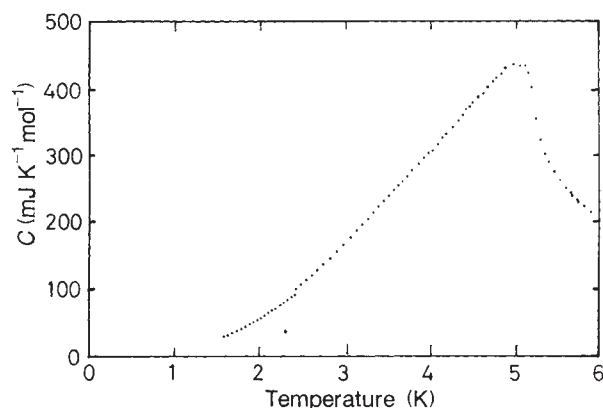


FIG. 3 The low-temperature specific heat of a cylindrical 422 sample in the range 1.5–6 K, determined using the ordinary adiabatic d.c. method.

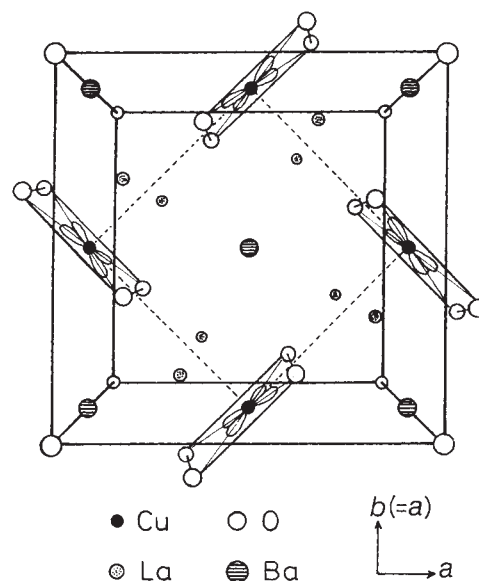


FIG. 4 Top view of the crystal structure of $\text{La}_4\text{Ba}_2\text{Cu}_2\text{O}_{10}$ showing the orthogonality of the wavefunctions of the Cu^{2+} d hole.

same plane and the nearest-neighbour distance between them is 4.84 Å.

The magnetic properties of non-stoichiometric samples, $\text{La}_{4-2x}\text{Ba}_{2+2x}\text{Cu}_{2-x}\text{O}_{10-2x}$, indicate that the ferromagnetism is abruptly destroyed at $x = 0.2$, consistent with the result of Michel *et al.*⁴, and that the oxygen-deficient site occurs in the CuO_4 planes. These results indicate an intimate relationship between ferromagnetism and the CuO_4 planes. When the structure in the planes is complete, the ground-state wavefunctions of the Cu^{2+} d holes are $z(x-y)$ and $z(x+y)$ at alternate sites. This configuration is very similar to the case of the two-dimensional ferromagnet K_2CuF_4 (ref. 7).

The relationship between $\text{LaBa}_2\text{Cu}_3\text{O}_7$ and $\text{La}_4\text{Ba}_2\text{Cu}_2\text{O}_{10}$ is homologous to that between $\text{YBa}_2\text{Cu}_3\text{O}_7$ and Y_2BaCuO_5 . The highest critical current density, $> 10^5 \text{ A cm}^{-2}$ at 77 K and 0 T, in bulk superconducting $\text{YBa}_2\text{Cu}_3\text{O}_7$ has been obtained by Murakami *et al.*⁸, and the impurity phase Y_2BaCuO_5 dispersed in this material proposed to play an important part in providing flux-pinning centres. We suspect that $\text{La}_4\text{Ba}_2\text{Cu}_2\text{O}_{10}$ may have a similar role in $\text{LaBa}_2\text{Cu}_3\text{O}_7$, and that the ferromagnetism may enhance its potential for flux pinning.

Moreover, a microwave frequency-mixing device has been developed recently that uses a superconductor-ferromagnetic insulator-superconductor heterostructure. The I - V characteristic of this device is controlled by quasiparticle tunnelling. The ferromagnetic insulating layers suppress the superconducting current (due to Cooper-pair tunnelling), which is the main cause of noise in the device⁹. There have been attempts to develop such a device using oxide superconductors and conventional ferromagnetic insulators. We anticipate that the properties of the $\text{La}_4\text{Ba}_2\text{Cu}_2\text{O}_{10}$ phase may be pertinent to such applications. □

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Effect of number of arms on diffusion of star polymers

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UNLIKE small-molecule liquids, polymer melts can display complex and often highly non-newtonian flow behaviour, which reflects the dynamics of the entangled polymer chains. Star-branched polymers, which have f arms each containing N_a monomeric units, joined covalently at a common branch point, provide good model systems for understanding the slow dynamics of branched polymers. These dynamics may be probed by measurement of the diffusion coefficient, D . It is well established¹⁻³ that for three-armed star polymers, $D \propto \exp(-\alpha N_a)$, where α is a constant. This result is consistent with early theories^{4,5}, which assumed that to diffuse, all but two arms of the star must retract simultaneously to the branch point; these theories predict $D \propto \exp(-\alpha(f-2)N_a)$. Here we present experimental measurements of D for polymers with $f=3-12$, which indicate a much weaker decrease with f : $D \propto \exp(-0.41f)$. This result is consistent with more recent theories⁶ which predict that a star can diffuse by retracting just one arm at a time.

Linear deuterated polystyrene precursor chains were synthesized by anionic polymerization of deuterated styrene monomer. These precursor chains were characterized by a weight-average degree of polymerization of 530 and a polydispersity index of 1.02, as determined by size-exclusion chromatography. Ter-

minating agents with functionalities of 3, 4, 6, 8 and 12 were added to separate portions of the linear precursor solution to produce star molecules with these numbers of arms per molecule. Several butadiene monomer units were added to the ends of the linear precursor chains to enhance the rate at which the coupling reactions take place. An excess of star arms was used so that each linking agent was fully saturated with arms, and these excess arms were separated from the star molecules by fractional precipitation. A complete description of the synthetic procedure is given elsewhere⁷. The only difference between the different star molecules is the number of arms per molecule, so these polymers are ideally suited for studies of the effects of arm number on polymer diffusion.

We measured the diffusion coefficients for the various star melts in matrices of high-molecular-weight linear chains using forward recoil spectrometry. This technique gives a direct measure of the volume fraction of the deuterated component as a function of depth in thin-film diffusion couples^{8,9}. Diffusion couples for this study were made such that the deuterated star polymers existed initially in a thin (~ 200 Å) layer between thicker ($\sim 4,000$ Å) layers of linear polystyrene chains. The molecular weights of the linear chains were high enough (2,000,000 or 4,000,000) so that there was no matrix contribution to the diffusion coefficient. The diffusion couples were annealed at high temperatures (178 °C or 184 °C) for times long enough for the distribution of deuterated material to broaden to a width of 3,000 Å or greater. Diffusion coefficients were obtained by fitting the depth profile of the deuterated volume fraction to the solution of the diffusion equation for a constant diffusion coefficient.

Diffusion coefficients of three-arm polystyrene stars in matrices of material with a high molecular weight have been measured previously³ for N_a as high as 420. These results are shown in Fig. 1, along with the measured diffusion coefficient from this study for a three-arm star with $N_a = 530$. Our measured value is consistent with the previously determined exponential dependence of D on N_a , $D \propto \exp(-\alpha N_a)$ with $\alpha = 0.0193$. The diffusion coefficients for the stars with $N_a = 530$ are plotted as a function of f in Fig. 2. The diffusion coefficients shown in Figs 1 and 2 correspond to a diffusion temperature of 178 °C, and were calculated from the measured values using the known temperature dependence of polystyrene diffusion^{10,11}. The deuterated tracer chains do not form an ideal solution with the hydrogenated matrix chains¹², giving rise to a thermodynamic slowing down of the diffusion process for finite concentrations of the star polymer¹³, which is further enhanced by the presence of butadiene segments at the star centres. We have corrected our data for the thermodynamic effect. This contribution becomes more important for higher tracer molecular weights and causes the concentration profile to become non-fickian. Our estimated uncertainty in fitting this profile is responsible for the larger error bars at the higher values of f . Within the uncertainty the data are described by the exponential law $D \propto \exp(-0.41f)$ shown as the solid line in Fig. 2.

A jump distance a and a jump frequency Γ can be defined such that $D = \Gamma a^2/6$. Early theories of star diffusion were based on the assumption that net motions of the branch point require simultaneous retractions of all but two of the star arms^{4,5}, giving $\Gamma \propto \exp(-\alpha(f-2)N_a)$ and $D(f) \propto a^2(f) \exp(-10.3f)$ for $\alpha = 0.0193$ and $N_a = 530$. This strong exponential dependence of D on f has been ruled out by a previous experimental study¹⁰, and is clearly at odds with our results for any reasonable dependence of a on f , as shown by comparing the dotted line with the data in Fig. 2. The observed f dependence of the diffusion coefficient can be explained more accurately from two related viewpoints. The first is that the branch point can move in a randomly spaced array of constraints each time a single arm retracts, in which case $\Gamma \propto f$ and the observed f dependence of the diffusion coefficient is determined by the f dependence of the jump distance. Our data are consistent with $a \propto f^{-1.8}$ if $\Gamma \propto f$.

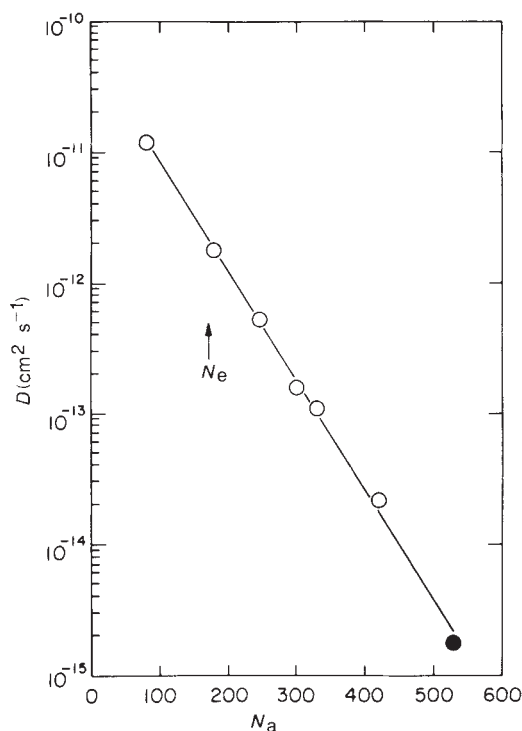


FIG. 1 Diffusion coefficients for three-arm polystyrene stars in high-molecular-weight matrices at 178 °C as a function of N_a , the degree of polymerization per arm. The data fall on the exponential curve, $D = 5.8 \times 10^{-11} \exp(-0.0193 N_a) \text{ cm}^2 \text{ s}^{-1}$, represented by the solid line. The open circles represent values reported in ref. 3, and the filled circle represents our value. The entanglement degree of polymerization for polystyrene¹⁴, $N_e = 173$, is also shown.

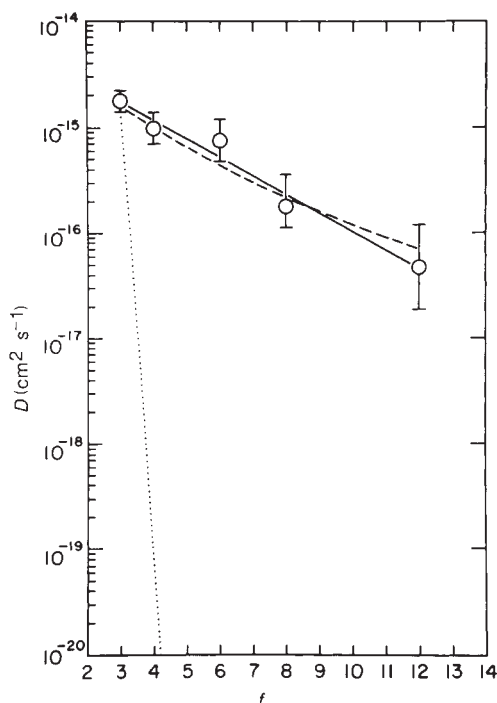


FIG. 2 Diffusion coefficients of f -arm polystyrene stars with $N_a=530$ in high-molecular-weight matrices at 178 °C. The solid line represents the exponential dependence, $D_0(f) \propto \exp(-0.41f)$, and the dotted line represents the exponential dependence predicted by the simultaneous arm retraction model, $D_0(f) \propto \exp(-10.3f)$. The dashed line represents the fit to equation (1), with $z=6$ and a not a function of f .

If we assume a regular lattice of constraints, a is not a function of f , and then $D_0(f)$ is proportional to Γ . Rubinstein⁶ obtained an expression for $\Gamma(f)$ by assuming that the entanglement network about the branch point defines z different 'gates' through which arms can emanate. The f dependence of the diffusion coefficient is obtained by assuming that diffusive steps can occur only when at most two of these gates are occupied by star arms

$$D_0(f) \propto a(f)^2 \frac{[1 + (z-1)(f-1)/2]z!f!}{(z+f-1)!} \quad (1)$$

The best fit to our data, assuming that a is not a function of f , is for $z=6$, corresponding to a cubic lattice. This prediction is shown as the dashed line in Fig. 2. Our data cannot be used to distinguish further between these two related models for star diffusion. □

A novel form of filamentous graphite

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CARBONACEOUS materials exist in many forms, with structures varying in the degree of structural order from diamond and hexagonal graphite to the less-ordered chars, soots and coals. Traditionally, graphite is produced from the less-ordered forms of carbon by exposing them to severe conditions of high temperature and pressure. Recently, there has been much interest in preparing highly ordered phases under relatively benign conditions. For example, diamond has been synthesized at low pressures^{1,2}, and carbon deposition on transition-metal catalysts has been shown to produce highly graphitic phases in a variety of morphologies^{3,4}. Here we show that disproportionation of carbon monoxide ($2\text{CO} \rightarrow \text{C} + \text{CO}_2$) catalysed by small iron-containing particles gives rise to a remarkably continuous stacking of fine graphitic layers on flat surfaces of the particles, producing carbon filaments with a ribbon-like morphology. Transmission electron microscopy reveals that stacking of the layers is not only very ordered but also unusual in being orientated perpendicular to the ribbon surface. These filaments may provide unique opportunities for studies of surface adsorption, catalysis and intercalation.

The carbon deposits obtained from hydrocarbon decomposition or CO disproportionation catalysed by iron-group metals exhibit a wide variety of morphologies including graphite flakes or platelets, lamellar graphitic crystallites and filamentous deposits of varying degrees of crystallinity^{4,5}. But some possible morphologies and/or structures have not so far been confirmed. The carbon filaments that we report here have a ribbon-like morphology and a microstructure different from those of hydrocarbon-derived vapour-grown carbon fibres or conventional CO-derived filaments, which have a cylindrical hollow-cored, twisted, or otherwise hollow morphology⁶⁻¹¹. Although ribbon-like filaments have been reported before¹², most of the products obtained in that work consisted of carbon shells or twisted carbon filaments. Until now the microstructure and properties of the ribbon-like filaments had not been determined and the catalyst particles had not been identified. In our search for highly ordered carbon deposits, we have recently investigated the

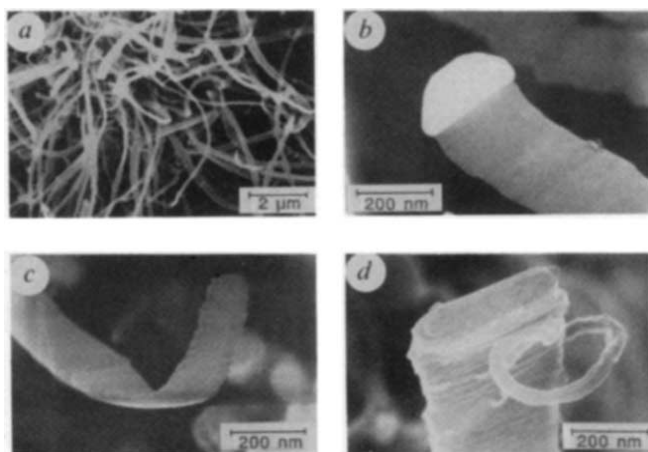


FIG. 1 Scanning electron micrographs of the ribbon-like filaments of graphite. *a*, Appearance of aggregated filaments prepared at 700 °C. *b*, A 'head' of a ribbon-like filament prepared at 700 °C. A bright particle is located on the tip of the filament and has nearly the same width as that of the filament. *c*, A 'tail' of a ribbon-like filament prepared at 700 °C. *d*, A cross-section of a ribbon-like filament prepared at 550 °C.

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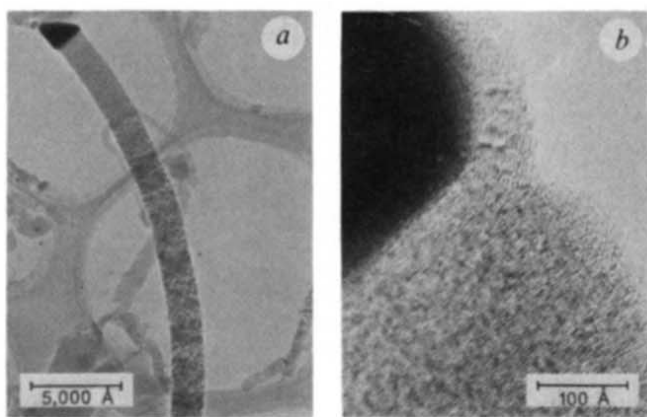


FIG. 2 Transmission electron micrographs. *a*, The appearance of a filament prepared at 700 °C. *b*, An enlargement of *a*, in which the lattice image of the filament and the interface between the catalyst and the filament can be seen.

Boudouard reaction and have found that the carbon deposition produced filamentous graphite under relatively benign conditions.

The filaments were prepared in the temperature range 400–700 °C using a thoroughly premixed continuous flow of CO/H₂ gas, containing Fe(CO)₅ for *in situ* generation of catalyst particles. A representative reactant gas composition is 49.6 vol% CO, 49.6 vol% H₂ and 0.8 vol% Fe(CO)₅. The electron micrographs show that >50 vol% of the products are ribbon-like filaments. The products contained 80–90% carbon by weight, and the yield based on the carbon weight of CO was, for example, 32% in the reaction at 700 °C. The filaments were of the order of 10 µm long, and most had a nearly rectangular transverse cross-section 0.1–0.7 µm wide and 0.01–0.2 µm thick, but some had an ellipsoidal cross-section with similar size and aspect ratio. Figure 1*a* shows a representative low-magnification electron micrograph of the filaments, and Fig. 1*b–d* shows typical individual filaments. High-resolution electron microscopy showed that the filaments were not hollow (Fig. 2*a*), and that the carbon layers had a uniform orientation perpendicular to the filament axis, these layers being closely parallel to the single flat surface of the catalyst particle (Fig. 2*b*). Figure 3*a* shows the microstructure of the filament surface arising from the stacked open sheets of carbon layers. After 30 minutes of heat treatment at 2,800 °C in argon, the structure of the surface was converted into a frill-like structure; the carbon layers were folded every several layers and most of the free edge carbon atoms



FIG. 4 *a*, Transmission electron micrograph of a ribbon-like filament prepared at 700 °C. *b*, Electron diffraction patterns from the catalyst particle and the carbon layers of the filament in the bright circle of *a*. The microdiffraction patterns from the catalyst particle are indexed as Fe₃C. The carbon layers exhibit a well defined pattern characteristic of graphite, clear evidence for the high degree of graphitization of the ribbon-like filament. The spots line from (103) Fe₃C and that from (002) graphite are observed concurrently in the same direction. *c*, Schematic diagram of the ribbon-like filament.

disappeared (Fig. 3*b*).

One of the most significant features of the filaments reported here is the high degree of graphitization obtained at a low deposition temperature. The pristine filaments grown at 700 °C, for example, have an average *d*₀₀₂ spacing of 3.366 Å, from which the degree of graphitization, as estimated from the equation of Mering and Maire¹³, is 86%. This is comparable to that of graphitized conventional vapour-grown carbon fibres heat treated at >2,500 °C (ref. 14). These ribbon-like filaments can be regarded as graphite flakes orientated in an unusually continuous array catalysed by specific iron-containing particles, and it is remarkable that the stacking of the graphite layers continues to lengths of the order of 10 µm.

The catalyst particles at the tip of the each filament had a single planar facet on which the continuous stacking of graphite layers took place, although the particles can be various shapes such as triangles, trapezoids and other polygons having more than five sides, including nearly semicircular shapes, as shown in Figs 1*b* and 2*a*. The thermal decomposition of Fe(CO)₅ in the gas phase usually yields spherical particles, for which the surface energy is a minimum. Thus the shape of the particles in this study seems to be controlled by the reactions of iron with CO and H₂ during the particle growth. The electron diffraction patterns (Fig. 4*b*) indicate that the catalyst particles are Fe₃C, and the interface between the particle and the carbon layers was identified as (103) Fe₃C/(002) graphite. A schematic diagram of the filament is depicted in Fig. 4*c*. The (103) plane of Fe₃C is the facet on which only iron atoms appear. There is no conformity, however, between the positions of iron atoms on (103) Fe₃C and that of the carbon atoms on (002) graphite. Nevertheless, the filaments no longer grow after the dissolution of the particles in HCl, so the particles seem to be the catalyst responsible for the filament growth.

Most of the highly ordered graphitic materials so far prepared have been films or filaments in which graphite layers are oriented parallel to their surfaces. The filaments reported here are highly ordered materials in which the graphite layers are oriented perpendicular to their large surfaces. Assuming that the growth mechanism of the ribbon-like filaments is operative over a wide size range, the synthesis of a new family of graphitic substances may be possible through careful design of the catalyst materials. These ribbon-like filaments have a large surface area (~110 m² g⁻¹) and numerous edges of graphite layers on the surface. Furthermore, the surface can be modified chemically so that it has many functional groups, or thermally so that it has neither functional groups nor free edge carbon atoms. Therefore, the filaments might be useful for catalyst carriers, hosts for intercalation compounds and as adsorbents. □

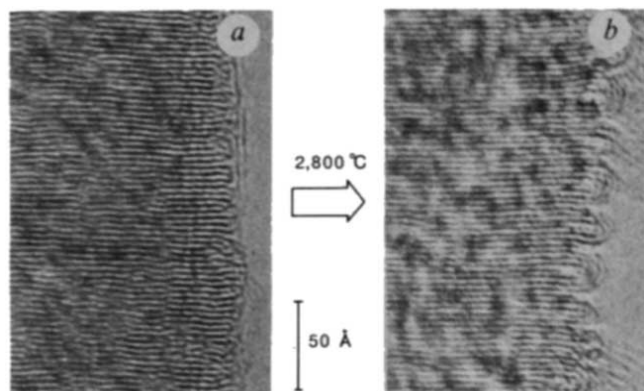


FIG. 3 Transmission electron micrographs showing the features of carbon layers at the edges of the ribbon-like filament. The growth direction is upward. *a*, In the pristine form prepared at 700 °C. *b*, After heat treatment at 2,800 °C in an argon atmosphere for 30 min.

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Remote sensing of ocean wave spectra by interferometric synthetic aperture radar

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THE two-dimensional power spectra of ocean waves are of great interest not only to oceanographers but also in practical applications such as wave forecasting, trans-oceanic ship routing, and design of coast and offshore installations. Remote sensing of ocean surface waves can be difficult using conventional synthetic aperture radar (SAR) techniques, but waves can be observed clearly by SAR in the interferometric configuration (INSAR)^{1,2}. This improvement is due to the ability of INSAR to provide images of the local surface velocity field, in contrast to conventional SAR for which the imaging process is related indirectly to the complex modulation of the surface reflectivity by longer waves and currents. Here we show that INSAR can be used to obtain wavenumber spectra that are in agreement with power spectra measured *in situ*. This new method thus has considerable potential to provide instantaneous spatial information about the structure of ocean wave fields.

The theory of microwave backscattering from a slightly rough surface was developed by Rice³. The dominant reflection for a side-looking radar is attributed to the resonance between the incident electromagnetic wave and the undulating surface. The resonant, or Bragg, condition is fulfilled when the crests of the surface waves are perpendicular to the radar viewing direction and the horizontal projection of the radar wavenumber is twice that of the resonant surface waves. Using a stationary Doppler radar, Crombie⁴ observed this frequency shift from a moving ocean surface. For microwave radar, the resonant Bragg waves

are relatively short (24.5 cm for L-band radar at 30° incidence angle). These waves are affected by the longer ocean surface waves and by currents, making the detection of the large-scale varying ocean features possible.

SAR is a high-resolution coherent remote sensor carried either by an aircraft or by an orbiting platform. The along-track (or azimuth) resolution is achieved by using the phase history of the Doppler-shifted return signal over a finite integration time in which the moving radar forms a large aperture in the azimuth direction. This Doppler shift is due to the line-of-sight component of relative velocity between the target and the moving radar. The cross-track (or ground-range) resolution is obtained by modulating the transmitted signal^{5,6}.

SAR imaging of a stationary area, as well as rigid moving targets, is well understood⁷. The imaging mechanism of a continuously varying ocean surface seems to be much more complicated and has been studied extensively for the past two decades^{8,9}. Hasselmann *et al.*⁹ have clarified some of the fundamental principles governing the backscattering and SAR imaging of the ocean. The indirect nature of the SAR imaging mechanism of the ocean surface is, in our view, the main difficulty in correlating the radar return signal with the actual ocean reflecting surface and is therefore an obstacle to obtaining quantitative information about the dominant ocean wave systems.

A modification of the conventional SAR sensor was suggested by Goldstein and Zebker¹ and Goldstein *et al.*². The technique consists of aligning two physically separated antennas located along the platform flight path. The backscattered echoes received by each antenna from the ocean surface are recorded and processed into two separate complex (magnitude and phase) maps. These maps are combined interferometrically into a single image. The SAR image of the ocean is a map of power reflectivity only, whereas each pixel in the INSAR image represents the line-of-sight velocity component of the surface. Such velocity is due to advecting currents, orbital velocity of long waves, and the phase velocity of the resonant waves. The INSAR image is thus directly related to the local scene by being proportional to the surface velocity. The ability of INSAR to sense ocean currents was demonstrated in refs 1 and 2. Here we show the potential of INSAR in imaging the complex ocean wave fields.

On 8 September 1989 remote imagery of the ocean surface in the nearshore region of Monterey Bay was carried out using an INSAR configuration installed on a DC-8 aircraft. The target area was ~12 × 6 km, centred on a wave pressure sensor located offshore at a depth of 16 m. The flight direction was northbound, roughly parallel to the shoreline. The dominant wave propagation direction towards the shore was thus close to the radar line-of-sight. The environmental conditions were favourable, with light westerly winds of <4 knots and significant wave height <0.6 m.

SAR and INSAR images are shown in Fig. 1. The size of a pixel is 6.0 m in the range (x) direction and 12.1 m in the azimuth (y) direction in both images. The flight direction was from right to left along the top of the image. The clearly visible coastline in the SAR image (Fig. 1a) separates the image into two different domains; the upper part clearly shows details of the countryside. The rest of the image represents moving ocean surface. In addition to the faint dominant waves, there are substantial local variations in the image intensity. These variations are probably

TABLE 1 Characteristics of the dominant waves

Area	Mean depth (m)	L_1 (m)	INSAR spectral estimates			Estimates from <i>in situ</i> frequency spectra	
			L_2 (m)	α_1	α_2	L_1 (m) ($T=15.9$ s)	L_2 (m) ($T=9.1$ s)
a	40	283	128	255°	292°	281	125
b	19	197	107	258°	285°	206	105

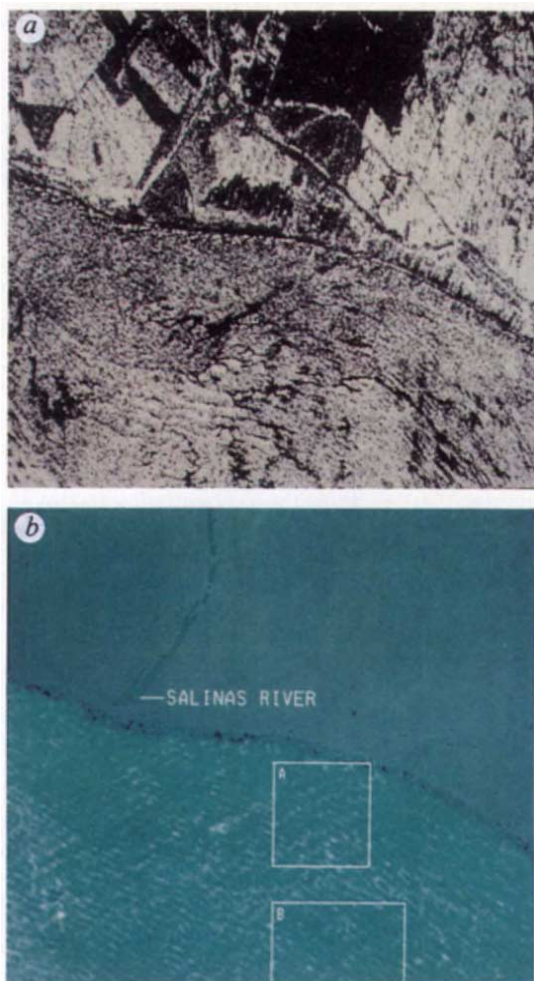


FIG. 1 Image of Marina Beach region in Monterey Bay. *a*, Conventional SAR image. *b*, Interferometric SAR image representing the velocity field.

associated with various ocean features, like internal waves or Langmuir cells^{11,12}.

In the interferogram shown in Fig. 1*b*, the difference between the stationary coast and moving water surface is enhanced significantly in comparison with the SAR image. The image of the coast is nearly uniform with the expected zero velocity, except for Salinas River. As seen in the image, the river is blocked off from the ocean at this time of year, and constitutes a basin of standing water in which waves are generated only by local wind. The apparent non-zero, positive surface velocity in

this basin is in agreement with the phase velocity of the upwind resonant waves ($\sim 50 \text{ cm s}^{-1}$ for typical inclination angles), as calculated from linear theory.

A bimodal wave system, which is barely observed in the SAR image (Fig. 1*a*), is clearly seen in the INSAR image (Fig. 1*b*). The long-crested swell experiences refraction and shoaling as it approaches the shore. The shorter swell propagating to southeast is less sensitive to depth variations; some refraction is visible in regions of larger bathymetric gradients.

The well-identified wave patterns in the INSAR image allow us to obtain meaningful local two-dimensional wavenumber spectra of small subregions of the dominant wave propagating over a sloping bottom. Two domains along the coastline with nearly uniform depths were selected (Fig. 1*b*). The size of the deeper water domain (mean depth $H_a = 40 \text{ m}$) is $3,000 \times 1,400 \text{ m}$. The second shallow domain, with $H_b = 19 \text{ m}$, is $2,200 \times 1,200 \text{ m}$. The two-dimensional wavenumber spectra for both domains are presented in Fig. 2. The mean line-of-sight velocity component, associated with surface currents and Bragg-wave phase velocity, has been removed. Two peaks can be identified in both spectra. The less energetic peak at lower wavenumber is well pronounced, whereas the energy density at higher wavenumber is more spread out. The 180° direction ambiguity, typical to all instantaneous spatial images, appears in the INSAR spectra as well. Here this ambiguity is resolved because waves naturally propagate towards the shore. The scanning distortion¹³ due to the relative velocity between the wave pattern and the radar platform is negligible for our experimental conditions.

Shoaling and refraction of waves can be characterized by the two-dimensional $\mathbf{k} = (k_x, k_y)$ spectra. Wavelengths L and propagation directions α corresponding to the peaks of Fig. 2 are summarized in Table 1. Both waves experience shoaling, becoming shorter with decreasing water depth. The variation of the wavelength with depth should satisfy the linear shoaling relation¹⁴

$$L_b \tanh(k_a H_a) = L_a \tanh(k_b H_b) \quad (1)$$

The results of Table 1 are in good agreement with equation (1). Refraction is more pronounced for the longer wave, where its relative water depth kH is shallower. The effect of wavenumber alignment towards the direction of the bathymetry gradient, $\alpha \approx 270^\circ$, is noticed for both wave systems.

The results obtained by the remote-sensing technique are verified by the *in situ* measurements. The surface-elevation power spectrum obtained by the wave sensor is shown in Fig. 3. The two peaks in the spectrum correspond to wave periods of $T_1 = 15.9 \text{ s}$ and $T_2 = 9.1 \text{ s}$. The wavelengths corresponding to these periods (Table 1) are calculated at the appropriate depths using the linear dispersion relation

$$\omega = 2\pi/T = (kg \tanh(kH))^{1/2} \quad (2)$$

where ω is the frequency and g is the acceleration due to gravity.

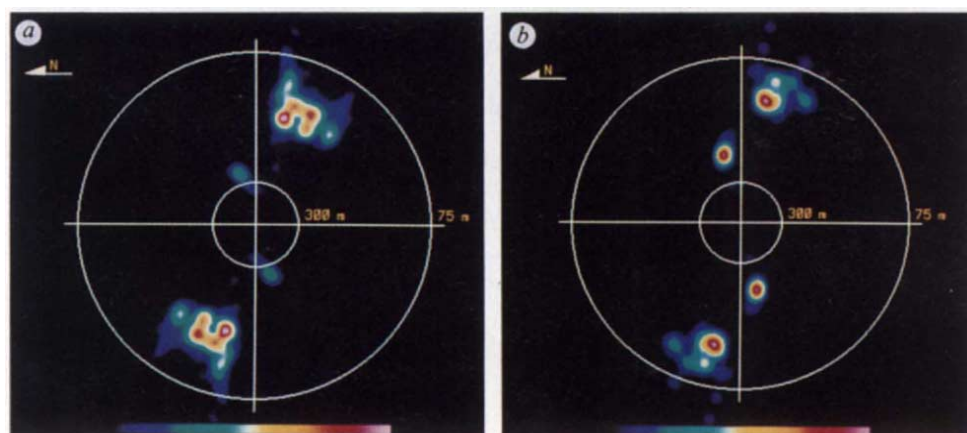


FIG. 2 Two-dimensional power spectra of the velocity field; calculated from subregions shown in Fig. 1*b*. *a*, Mean depth of 19 m. *b*, Mean depth of 40 m.

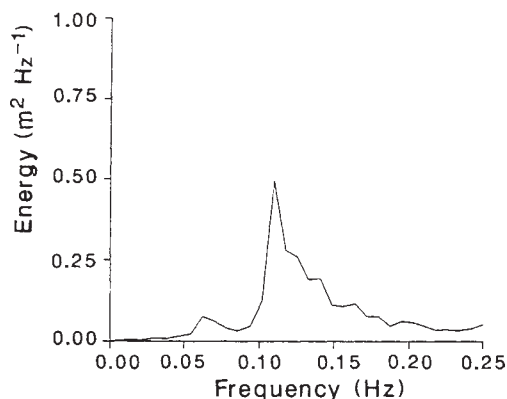


FIG. 3 *In situ* frequency power spectrum of surface elevation measured in the imaged area at the depth of 16 m.

These results are in good agreement with the dominant wavelengths estimated from Fig. 2.

The observed wave systems were mainly range orientated. This orientation is favourable for radar imagery because it minimizes image distortion due to the azimuth image shift (velocity bunching)⁹. In the conventional SAR, velocity bunching is often a dominant imaging mechanism. INSAR image formation, however, is not based on this mechanism. In the image of the same area obtained in an orthogonal flight path, which will be reported elsewhere, ocean waves are still visible

by INSAR, but are not seen in conventional SAR image. This indicates that the range orientation is probably not essential for obtaining clear INSAR images of ocean waves.

Together with earlier works^{1,2}, which demonstrated INSAR capabilities in measuring ocean surface currents, our work reveals the considerable ability of this novel technique to provide reliable quantitative spatial information about dynamic processes at the ocean surface. Realization of the potential of this technique with spacecraft is unfortunately not straightforward but might lead to the monitoring of global ocean wave spectra and surface currents. □

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Evidence for thinning of the Arctic ice cover north of Greenland

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IN May 1987 a British submarine carried out an ice profiling experiment in the Arctic Ocean in which the route closely approximated that of an earlier voyage in October 1976. Over a zone extending more than 400 km to the north of Greenland there is evidence of a significant decrease in mean ice thickness in 1987 relative to that found in 1976. This thinning amounts to a loss of volume of at least 15% over an area of 300,000 km². The nature of the ice thickness distributions suggests that the thinning is primarily associated with the presence of a larger fraction of young and first-year ice, a consequence of wind-driven divergence in the ice cover during the months preceding the 1987 observations. Such a large fluctuation in a region previously believed to possess consistently high ice thicknesses illustrates the importance of monitoring Arctic ice thickness more systematically to determine whether such fluctuations fall within the limits of normal inter-annual variability.

The 1987 expedition involved a submarine and two remote-sensing aircraft. Results from various sensors are reported in refs 1–3. The submarine carried a 48-kHz upward-looking echo sounder of narrow beamwidth (<5°) and obtained a profile of total length ~6,000 km from the Greenland Sea and the Arctic Ocean. Data were recorded digitally and on a paper chart, which was used for interpolation whenever the digitizer lost lock. Data points were obtained at constant time intervals (~0.25 s) and so, to obtain a uniformly spaced record, the profile had to be corrected for varying submarine speed. Depth variations were

corrected by constructing a smoothed profile using data from open water (open leads and cracks) encountered along the track and subtracting it from the ice profile. The resulting draft values were interpolated to a standard interval of 1 m and statistically analysed in 50-km sections. Statistical results obtained in earlier cruises⁴, in which several transects were obtained in the same region, suggest that the repeatability in mean ice draft estimated over a 50-km interval is ±0.06 m.

The results are compared with a record obtained by another British submarine in October 1976, using an echo sounder of similar type and frequency but with a transducer of wider beamwidth⁵. Data from both the 1976 and 1987 voyages were collected by the author. Figure 1a shows contours of mean ice draft in 1976 constructed from values calculated over 100-km track sections, corrected for beamwidth as described in ref. 5. Figure 1b shows contours of mean ice draft from the 1987 data, constructed from values calculated over 50-km track sections, uncorrected for beamwidth. These results are conservative because a beamwidth correction to the 1987 data would produce a further reduction in apparent mean draft of some 1–2%. This small effect should be borne in mind with respect to the figures quoted below.

The significant differences between the two maps lie in the region extending northwards from Greenland to the North Pole (0–90° W at 82–90° N); in the eastern European Arctic (east of 0°) there were no significant differences between the mean drafts. The 1976 map shows a steady build-up of mean ice thickness as the coast of Greenland is approached. This effect was predicted in a numerical model⁶ on the basis of the overall long-term pattern of wind-driven surface circulation in the Arctic Ocean. This consists of two main systems, the Beaufort Gyre in the Canada Basin and the Trans Polar Drift Stream in the Eurasian Basin. In both currents ice is driven from the general region of the North Pole towards the coasts of Greenland and Ellesmere Island. At a latitude of ~87° N the ice begins to feel the influence of the coast, transmitted as internal stress through the ice from the downstream land boundary. This causes pressure ridges to

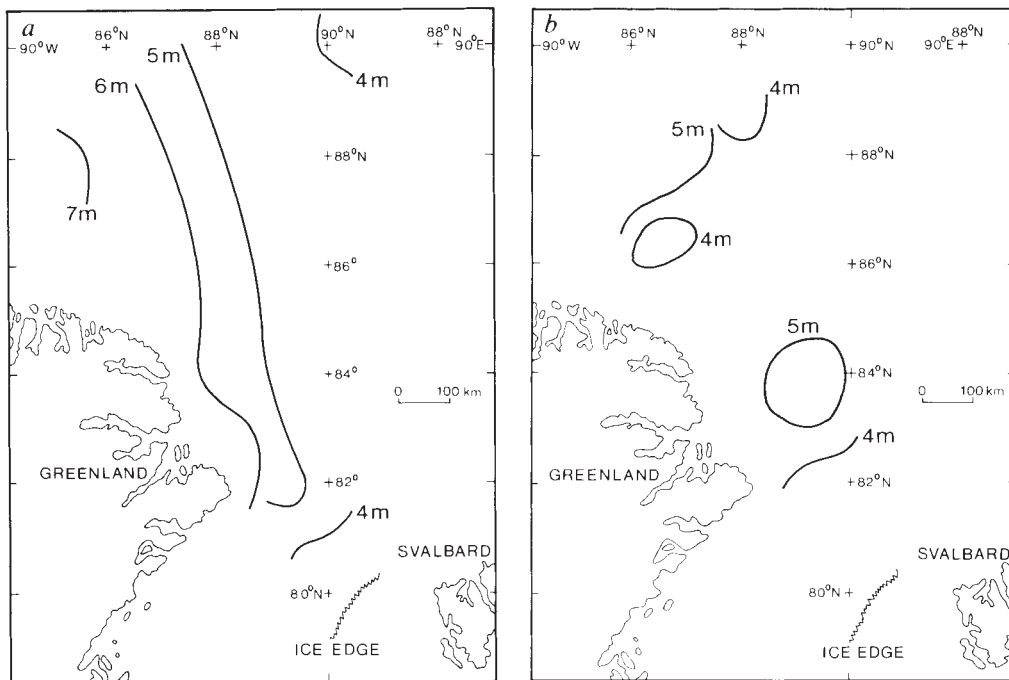


FIG. 1 *a*, Contour map of mean ice drafts from British submarine cruise, October 1976. *b*, Contour map of mean ice drafts from cruise of May 1987.

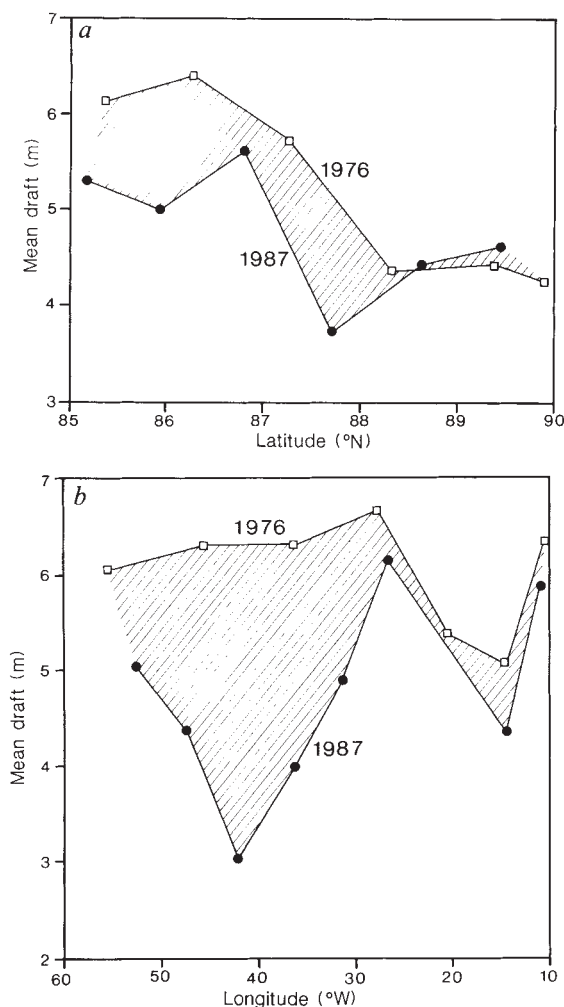


FIG. 2 *a*, Comparison of mean ice drafts from 1976 and 1987 along a N-S transect from North Pole to 85°N. *b*, Comparison of mean ice drafts for transect across north of Greenland from 60°W to 10°W.

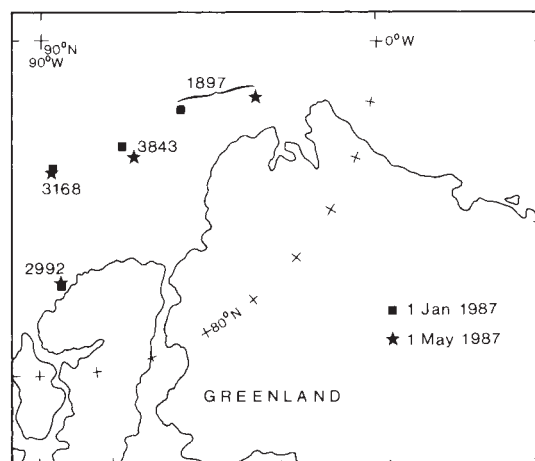
form from the crushing of refrozen leads containing young ice, producing an increase in mean ice draft.

In 1987 no such significant build-up seems to have occurred. I have assessed the magnitude of this change in two ways. To calculate the overall effect in the whole region, I considered a triangle bounded by the Greenwich meridian from 80°N to 90°N, the 70°W meridian from the North Pole to 85°N, and a great circle from 85°N 70°W to 80°N 0°W. The mean draft in this 290,000 km² area was 5.337 m in 1976 and 4.548 m in 1987. The decrease, 78.8 cm, represents a 14.8% loss of ice volume, or 229 km³. Inclusion of beamwidth effects would increase this loss to ~16–17%.

In the second approach I considered the actual mean ice drafts for corresponding sections of track. Figure 2*a* compares the mean drafts for 100-km track sections from 1976 measured along the 70°W meridian from the North Pole to 85°N with 100-km mean drafts from 1987 data measured along a meridian that was, at most, 78 km from the 1976 transect. Figure 2*b* shows a similar comparison between the 1976 data from 10°W to 60°W and corresponding 1987 data from a line that always lay less than 30 km from the 1976 transect. It can be seen from Fig. 2*a* that the loss of ice is concentrated in the region south of 88°N, and that even in 1987 some modest build-up of ice towards the Greenland coast did in fact occur. Figure 2*b* shows that in terms of longitude the loss is greatest in 40–50°W, in the 'dead' region where the drifting ice divides between an eastward flow into Fram Strait and a westward flow towards the Beaufort Sea. East of 30°W there is little change between the two years. Large spatial variations in mean ice thickness are known north of Greenland^{6,7}: in the region of interest, the east-west and north-south thickness gradients are ~1 m per 250 km and ~1 m per 90 km, respectively⁷. From these gradients, the maximum difference in mean ice thickness between 1976 and 1987 resulting from the track differences alone is 0.3 m in Fig. 2*a, b*, which is much less than the actual variations observed. The track differences, therefore, cannot by themselves explain the discrepancy between the 1976 and 1987 data.

There are only two other direct comparisons of data sets obtained in the same region of the Arctic Ocean in different years or seasons. I previously found⁸ that in the Trans Polar Drift Stream north of Fram Strait the mean ice thickness was

FIG. 3 Positions of drifting buoys north of Greenland on 1 January 1987 and 1 May 1987.



remarkably constant over measurements made in October 1976, April–May 1979 and June–July 1985. All three datasets came from Royal Navy submarine cruises and were recorded with a similar echo sounder. McLaren⁹ compared data from two US Navy submarine transects of the Arctic Ocean in August 1958 and August 1970, running from the Bering Strait to the North Pole and thence to Fram Strait. He found similar ice conditions in the vicinity of the North Pole and in the Eurasian Basin, but milder conditions in the Canada Basin in 1970, which may be due to anomalous cyclonic activity observed there in some recent summers¹⁰. Bourke and Garrett⁷ presented contour maps based on a compilation of data sets of varying quality, including semi-quantitative sources such as cruise reports by submarine commanding officers; the maps indicate a spring thinning north of Greenland, but the authors state that the thinning is “partly attributable to the sparseness of data available for this area”. Numerical models based on monthly averaged temperature and wind data⁶ predict that the build-up of thickness against the coast of Greenland is normally greatest in spring (April).

There are four main ways in which a change in the probability density function of ice thickness can result in a decrease in the mean value: a lesser degree of pressure ridging; a greater amount of open water or young ice in leads; a change in the proportions of different ice types present, with more first-year and less multi-year ice; a lower mean draft for undeformed ice of a given age. The last effect is a thermodynamic thinning which might be an indicator of ‘greenhouse warming’, whereas the first three would be caused by some radical change in the pattern of surface drift, itself an indicator of an important change in wind patterns over the Arctic during the period preceding the measurements. To distinguish between these effects I have considered the proportions of ice in different draft categories.

Table 1 shows the results from two corresponding track sections each 300 km in length from the southern end of the N–S transect of Fig. 2a, and two 200-km sections from the region 40–50° W in the E–W transect of Fig. 2b. In 1987 there was much more young ice in refrozen leads (coherent stretches of ice in which the draft is under 1 m), and first-year ice (usually <2 m thick), than in 1976. There was less multi-year ice (2–5 m

thick) and less ridging (represented by ice >5 m thick) in 1987 in the E–W transect although slightly more in the N–S transect. The ice deficit in 1987 therefore seems to be mainly due to a greater amount of young and first-year ice in the composition of the ice cover, rather than to a general thinning of all ice types.

Some insight into a possible cause of this can be obtained by examination of ice draft data from the satellite-tracked buoys of the Arctic Ocean Buoy Program (University of Washington). Figure 3 shows the positions of the four buoys that were present in the region in the months preceding the 1987 cruise, using data from ref. 11 and from R. L. Colony (personal communication). Whereas buoy 1897 drifted rapidly towards the Fram Strait in the Trans Polar Drift Stream, the array of 3843, 3168 and 2992 remained almost stationary between January and May 1987. This halting of the motion of the Beaufort Gyre throughout the winter is an anomaly, although temporary reversals of the gyre in summer owing to cyclone outbreaks have been reported^{10,12}. If the ice to the west of the region is stationary while the ice to the east is moving towards Fram Strait, open water is created, allowing young ice to grow.

These remarkable variations in ice thickness indicate the need for more extensive monitoring of Arctic ice thickness to determine whether the Arctic Basin as a whole is experiencing a real progressive decrease in overall mean ice thickness or whether fluctuations of this magnitude are merely interannual effects. Thinning of the ice and a decrease in its extent are expected to be an early consequence of ‘greenhouse warming’, the magnitude of which is predicted to be greatly enhanced in the Arctic regions relative to lower latitudes^{13,14}, but in the absence of data from intervening years or from years since 1987 it is not possible to confirm or deny an effect from global warming in these data sets. □

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TABLE 1 Comparisons of ice statistics from 1976 and 1987 datasets

	N–S transect		E–W transect	
	1976	1987	1976	1987
Mean draft (m)	6.09	5.31	6.32	4.07
Ice <2 m draft (%)	11.6	16.7	7.9	29.9
Ice 2–5 m draft (%)	48.7	38.6	46.5	39.6
Ice >5 m draft (%)	39.7	44.7	46.0	30.5
Refrozen leads (%)	4.0	7.9	3.7	15.6

A field experiment to test whether organic acids buffer acid deposition

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THE role of organic acids in surface-water acidification is a matter of recent controversy¹⁻⁸. It has been suggested^{1,4-6} that lakes and streams in the northeastern United States and southern Scandinavia that have high mineral acidity resulting from acid deposition had, before acid deposition, high concentrations of dissolved organic carbon (DOC) and were acidified by natural organic acids. The suggestion is that deposition of strong mineral acids has been buffered by concurrent losses in organic acids and DOC⁴⁻⁶, resulting in little or no overall change in pH. Despite considerable debate⁶⁻⁸ and comparative analyses of lake chemistry^{2,7}, this hypothesis has never been tested experimentally in the field. Here we present results from an experimental acidification of a brown-water stream that tests two of the major elements of the hypothesis. We find that DOC concentrations are not reduced by acidification, and that the organic acid-base system has only a very limited capacity to buffer inputs of strong mineral acids. In addition, mineral acids mobilize toxic forms of aluminium.

We acidified a small stream which drains watershed 9 (W9) of the Hubbard Brook Experimental Forest (HBEF) (New Hampshire, United States) to test two processes that have been suggested to be important in the acidification of surface waters: the weak-acid buffering by natural organic acids in surface water^{1,4} (which we will refer to as process A) and the flocculation of humic substances and loss of DOC from solution in surface waters^{1,4-5} (which we will refer to as process B). The stream-water DOC concentrations range from 7 to 15 mg C l⁻¹, reflecting the abundance of coniferous tree species, and shallow organic soils⁹; the pH ranges from 4.2 to 4.6. The channel consists of bedrock, and inorganic and organic sediment accumulations, which form alternating pool and riffle areas. This experiment differs from earlier acid additions to clear-water streams at HBEF¹⁰ and elsewhere^{11,12} in that concentrations of DOC and organic acids are higher in the W9 stream. The ambient pH in the W9 stream is in the range of average pK for organic acids (~3.5-4.5)^{13,14}, indicating that the capacity of organic acids to buffer inputs of strong mineral acids is high. Thus, the W9 stream conforms closely to the type of high-DOC, low-pH surface water in which the above two processes have been suggested to be of greatest importance⁴.

A concentrated solution of sulphuric acid (H₂SO₄) was added continuously for 20 h to the stream from a Mariotte bottle fitted with a Teflon-stem valve. The loading rate was adjusted to elevate the stream-water sulphate concentration by 150 µeq l⁻¹ above the background level. This value reflects a SO₄²⁻ increase equivalent to the present loadings in wet and dry deposition (adjusted for water losses owing to evaporation and transpiration) in the northeastern United States and southern Scandinavia^{15,16}. A known concentration of LiBr was added to the acid as a conservative tracer to allow discrimination, at any downstream site, between concentration decreases of added acid owing to chemical neutralization and concentration decreases owing to dilution by soil water or small tributaries (Fig. 1).

Water samples were collected at an upstream reference site (r) and at five downstream sites (13, 30, 45, 64 and 108 m below

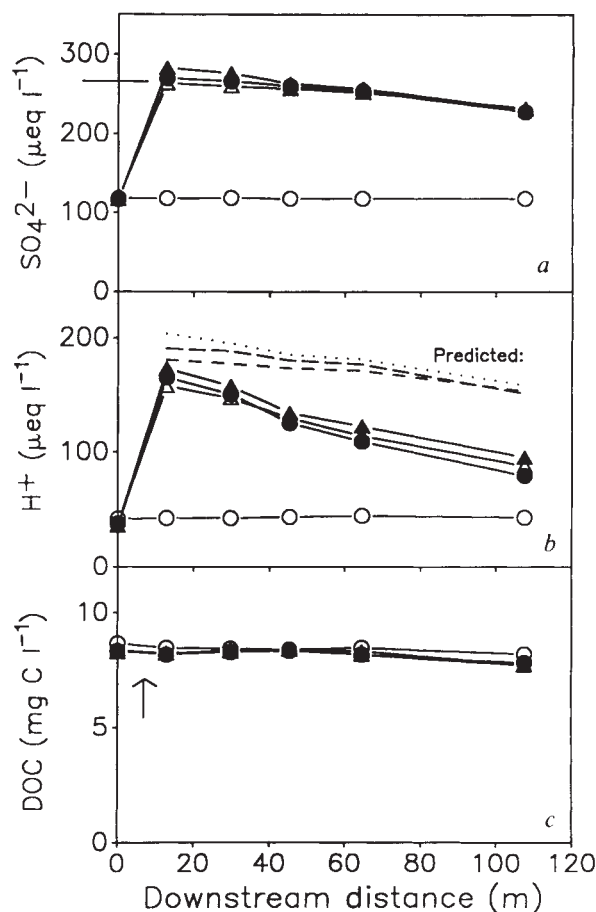


FIG. 1 Concentrations of SO₄²⁻, H⁺ and DOC before and during experimental H₂SO₄ addition to the W9 stream. Samples were collected before the addition (○) (N=2), and at 8 h (●) (N=1), 12 h (△) (N=2) and 20 h (▲) (N=1) after the start of the acid addition. a, SO₄²⁻: the horizontal line segment on the vertical axis indicates the target concentration of SO₄²⁻. b, H⁺: dashed lines indicate predicted concentrations of H⁺ based on dilution only (see methods); long-dashed line, 8 h; medium-dashed line, 12 h; dotted line, 20 h. c, DOC. The site of H₂SO₄ addition is indicated by the arrow.

METHODS. DOC samples were filtered through a pre-ashed, pre-rinsed Whatman GFF filter and analysed by the persulphate oxidation method²⁶, which provides low analytical variability (coefficient of variance <1% at 10 mg C l⁻¹; N=8) and an efficiency comparable to the commonly used Dohrmann method²⁶. The pH was determined potentiometrically at air equilibrium; Al³⁺ and Al-Org by ion-exchange fractionation and a colorimetric method²⁷. Other ions were determined by methods described in ref. 24. Average ion concentrations and standard deviations (N=2) at the reference site, immediately before the acid addition were: DOC, 8.7 ± 0.04 mg C l⁻¹; organic anions, 69.4 µeq l⁻¹, of which [A⁻] = 37.5 ± 1.1 µeq l⁻¹ and [Al-bound organic anions] = 31.9 ± 0.1 µeq l⁻¹; SO₄²⁻, 118 ± 0.1 µeq l⁻¹; NO₃⁻, <0.8 µeq l⁻¹; Cl⁻, 8.8 ± 0.1 µeq l⁻¹; Br⁻, <0.1 µeq l⁻¹; Ca²⁺, 41.5 ± 0.4 µeq l⁻¹; Mg²⁺, 21.6 ± 0.1 µeq l⁻¹; Na⁺, 34.3 ± 0.04 µeq l⁻¹; K⁺, 2.4 ± 0.1 µeq l⁻¹; NH₄⁺, 0.7 ± 0.05 µeq l⁻¹; Al³⁺, 22.1 ± 0.8 µeq l⁻¹; Al-Org, 31.9 ± 0.1 µeq l⁻¹; H⁺, 42.2 ± 0.7 µeq l⁻¹; laboratory DIC, 7.5 µM; field DIC, 45 ± 18 µM; dissolved Si, 61.1 ± 2.2 µM; F⁻, 1.5 ± 0.1 µM. Downstream dilution of the LiBr was used to calculate predicted SO₄²⁻ [Pred-SO₄] and H⁺ concentrations (based on dilution losses only) at site s [Pred-SO₄]_s = (R × [Br⁻]_s) + ([SO₄²⁻]_r × CF_{SO₄s}), where R = [SO₄²⁻]_r/[Br⁻]_r in addition solution; [Br⁻]_s is the Br⁻ concentration at site s; [SO₄²⁻]_r is the SO₄²⁻ concentration at the upstream reference site; CF_{SO₄s} is the longitudinal correction factor for site s based on the ratio [SO₄²⁻]_s/[SO₄²⁻]_r, observed before the acid addition. Thus, CF_s corrects for any slight downstream trends in ion concentrations (0.94 > CF > 1.06). Predicted H⁺ concentrations ([Pred-H]) were calculated as above, but using appropriate values for R ([H⁺]_r/[Br⁻]_r) and CF_{H+s} ([H⁺]_s/[H⁺]_r).

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r), refrigerated within 1 h and analysed for pH, DOC, dissolved inorganic carbon (DIC), labile monomeric aluminium (Al^{n+} , representing dissolved inorganic aluminium¹⁷) and nonlabile monomeric aluminium (Al-Org, representing organically bound aluminium¹⁷), Ca^{2+} , Mg^{2+} , Na^+ , K^+ , NH_4^+ , Li^+ , Br^- , Cl^- , NO_3^- , SO_4^{2-} , F^- and dissolved silica. The pH, DOC, DIC, Al^{n+} and Al-Org were analysed within 24 h, and NH_4^+ within 48 h (details on methods and analytical variability are given in Fig. 1). Aluminium speciation was calculated using the ALCHEMI chemical equilibrium model¹⁸, considering OH^- , F^- and SO_4^{2-} complexation. Concentrations of dissociated organic anions (A^-) were calculated, using the charge-balance approach¹⁹, as the difference between cationic and anionic equivalent charges.

Concentrations of SO_4^{2-} and H^+ increased markedly in the experimental reach following the H_2SO_4 addition (Fig. 1a, b). Immediately below the addition site SO_4^{2-} concentrations remained close to the targeted $150 \mu\text{eq l}^{-1}$ increase (Fig. 1a). Sulphate decreased slightly with downstream distance owing to dilution (Fig. 1a). The close agreement between actual concentrations of SO_4^{2-} and those predicted based on LiBr dilution (average difference in values, $0.3 \mu\text{eq l}^{-1}$; standard error (s.e.) = 0.07) indicates that other mechanisms of SO_4^{2-} loss (such as sorption) were not significant.

Concentrations of H^+ also decreased with downstream distance from the addition site (Fig. 1b), but the measured H^+ concentrations were markedly lower than concentrations predicted based on dilution alone (Fig. 1b), indicating substantial chemical neutralization of the added H^+ . Indeed, neutralization of the added H_2SO_4 was nearly 60% complete at the 108-m site (Fig. 1b).

Aluminium and base cations (C_B , $[\text{Ca}^{2+}] + [\text{Mg}^{2+}] + [\text{Na}^+] + [\text{K}^+]$) increased significantly in response to the H_2SO_4 addition, whereas A^- decreased. Before the manipulation, dissolved aluminium was largely in an organic form (Al-Org). After the addition of acid the concentration of inorganic aluminium (Al^{n+}) increased markedly and Al^{n+} became the predominant form of monomeric aluminium (Fig. 2). No other ions showed significant ($>1 \mu\text{eq l}^{-1}$) concentration changes. Neutralization of the added acid (in $\mu\text{eq l}^{-1}$) by (1) aluminium release (dissolution or exchange) (Al-buf), (2) base cation release (exchange or weathering) (CB-buf), and (3) organic anion protonation (OA-buf) at the farthest downstream (108-m) site were calculated as follows

$$\text{Al-buf}_{108\text{m}} = ([\text{Al}^{n+}]_{108\text{m}} - ([\text{Al}^{n+}]_r \times \text{CF}_{\text{Al},108\text{m}})) \quad (1)$$

$$\text{CB-buf}_{108\text{m}} = ([\text{C}_B]_{108\text{m}} - ([\text{C}_B]_r \times \text{CF}_{\text{CB},108\text{m}})) \quad (2)$$

$$\text{OA-buf}_{108\text{m}} = ([\text{Pred-H}^+]_{108\text{m}} - (\text{Al-buf}_{108\text{m}} + \text{CB-buf}_{108\text{m}} + [\text{H}^+]_{108\text{m}})) \quad (3)$$

where r is the reference site, $[\text{Pred-H}^+]_{108\text{m}}$ is the H^+ concentra-

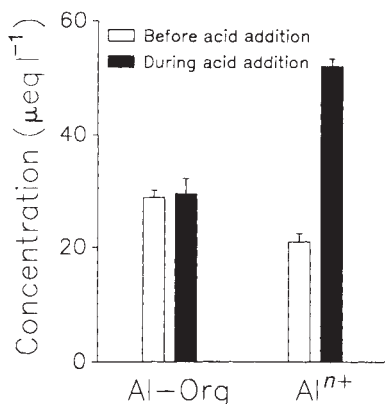


FIG. 2 Concentrations of organically complexed aluminium (Al-Org) and dissolved monomeric inorganic aluminium (Al^{n+}) at the 108-m site before ($N=2$) and during acid addition ($N=3$). Error bars indicate standard errors.

tion predicted based on dilution only (assuming no chemical reactions), and $\text{CF}_{\text{Al},108\text{m}}$ is the longitudinal correction factor for Al^{n+} at the 108-m site based on the ratio $[\text{Al}^{n+}]_{108\text{m}}/[\text{Al}^{n+}]_r$, measured immediately before the acid addition ($\text{CF}_{\text{Al},108\text{m}} = 1.0$; $\text{CF}_{\text{CB},108\text{m}} = 0.98$).

Aluminium release was the most important mechanism of acid neutralization, consuming $31.2 \mu\text{eq l}^{-1}$ of the added acid (s.e. = $0.80 \mu\text{eq l}^{-1}$; $N=3$ transects), followed by base cation release which provided a further neutralization of 19.4 (s.e. = 1.9) $\mu\text{eq l}^{-1}$ (Fig. 3). Organic anion protonation was the only other significant buffer mechanism, providing 16.7 (s.e. = 0.73) $\mu\text{eq l}^{-1}$ neutralization (Fig. 3). This *in situ* estimate of acid-neutralization capacity for organic anions in the W9 stream was not significantly ($P>0.5$; t -test) different from values predicted from laboratory titrations of W9 water ($16.0 \mu\text{eq l}^{-1}$; s.e. = 2.6; $N=2$) at added H_2SO_4 concentrations of $150 \mu\text{eq l}^{-1}$. Because such laboratory titrations do not consider neutralization processes by stream sediments, they overestimate the importance of pH buffering by the organic acid-base system relative to Al^{n+} mobilization and base cation exchange. Nevertheless, the overall capacity of organic anions to neutralize inputs of strong mineral acid to the W9 stream (process (A)) was limited, accounting for only ~11% of the added H_2SO_4 concentration of $150 \mu\text{eq l}^{-1}$.

It has been hypothesized⁴⁻⁶ that anthropogenic inputs of strong mineral acids result in marked decreases in DOC concentrations of surface waters, owing to the protonation of organic anions or co-precipitation with mobilized aluminium or Ca^{2+} (process (B)). But the experimental H_2SO_4 addition to the W9 stream did not result in any decrease in DOC (Fig. 1c) despite significant increases in Al^{n+} and Ca^{2+} , and in organic anion protonation. There was no significant effect ($p>0.19$; F -test) of the acid treatment on the downstream trend in DOC concentrations (Fig. 1c). In addition, aluminium associated with organic anions (Al-Org) did not increase in response to the acid addition despite the significant increases in Al^{n+} (Fig. 2). Finally, laboratory additions of H_2SO_4 (range $0-600 \mu\text{eq l}^{-1}$) to W9 stream water did not result in any loss in DOC from added H_2SO_4 ($P>0.38$; F -test) during incubations ranging from 1 to 12 days. Thus, we found no indication of decreases in DOC concentrations as a result of the addition of H_2SO_4 at $150 \mu\text{eq l}^{-1}$ to the W9 stream. Loss of DOC may be more important in waters with higher concentrations of Al^{n+} or Ca^{2+} , or where acid loadings are higher. Indeed, evidence for significant decreases in DOC has come from studies²⁰ in which unrealistically high treatment levels of mineral acids were

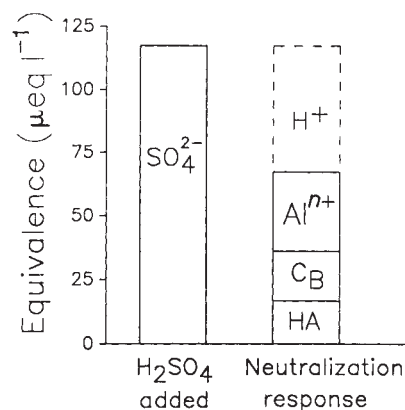


FIG. 3 Neutralization response of H_2SO_4 added to the experimental reach. The left bar shows the average ($N=3$) equivalence of SO_4^{2-} at the downstream (108 m) sampling site owing to the experimental H_2SO_4 addition. The right bar shows the average ($N=3$) experimental response (at the 108-m site) of different mechanisms in neutralizing the added H_2SO_4 . H^+ , added H^+ that was not neutralized in the experimental reach; Al^{n+} , neutralization by aluminium release; C_B , neutralization by base cation release; HA, neutralization by organic acid protonation, $\text{H}^+ + \text{A}^- \rightarrow \text{HA}$.

used. Other studies²¹ have found increased leaching of DOC and organic acids from soils owing to additions of mineral acids.

Our conclusions are supported by data from experimental manipulations of entire catchments at HBEF. Experimental deforestation resulted in substantial increases in the acidity of both catchment soils and stream water by mineral acid (HNO_3)^{22,23}. But this acidification did not result in loss of DOC in stream water^{22,23} or in soil solutions (organic and lower mineral soil horizons)²², whereas concentrations of inorganic aluminium and base cations increased markedly^{23–25}. Nevertheless, these catchment manipulations did not allow for the

detailed mechanistic examination of processes (A) and (B) above that was possible in the W9 manipulation.

The capacity of organic anions to buffer inputs of strong mineral acids probably increases as a function of DOC concentrations. Based on our results, we calculate that organic anions provided 2.0 μeq acid-neutralizing capacity per mg C of DOC. This estimate represents 10–14% of total organic anion sites present per mg C of DOC (~14–20 μeq per mg DOC) in the lakes of the northeastern United States^{14,19}. Thus, given an ecologically relevant increase in acid loading, only a limited fraction of total organic anion sites seem to contribute to the acid-neutralizing capacity. □

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Subcontinental mantle exposed in the Atlantic Ocean on St Peter–Paul islets

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ST PETER–PAUL (Penedos de São Pedro e Paulo), tiny islets in the Atlantic ocean just north of the Equator, have interested Earth scientists for more than a century. Darwin¹ was the first to recognize their peculiarity: he landed on St Peter–Paul (SPP) from the *Beagle* in 1831 and noticed that, unlike most oceanic islands, SPP are not volcanic. Subsequent studies confirmed Darwin's view, and established that SPP are made mostly of peridotites and represent an uplifted body of upper mantle^{2–7}. As a result, SPP have been cited frequently as providing a representative sample of sub-oceanic mantle. Here I argue that, although they are located on a major transform zone less than 200 km from the axis of the Mid-Atlantic Ridge, SPP are not a sample of sub-oceanic mantle. Mineral chemistry, elemental and isotopic chemistry and geothermometry suggest that SPP are a relict of sub-continental mantle, which was left behind during the opening of the equatorial Atlantic, and was then tectonically uplifted to its present position.

SPP is the exposed summit of an ENE-elongated relief striking parallel to and on the northern side of the St Paul transform zone⁸, which offsets the axis of the Mid-Atlantic Ridge (MAR) by ~200 km (Fig. 1). SPP exposes mylonitic spinel peridotites that contain variable quantities of amphibole, and are associated with hornblende^{2–7}. Similar rock types have been dredged from the submarine slopes of the SPP massif, and a fresh alkali basalt has been recovered at one site north of SPP^{9,10}.

The SPP peridotites contain primary mantle-equilibrated assemblages in the form of relict augen within the variously metamorphosed and mylonitized rocks. Samples SE9 and SE29⁷ contain such primary augen. This primary assemblage is made of olivine, orthopyroxene, clinopyroxene and spinel, suggesting

equilibration under pressure and temperature conditions of the spinel hercynite facies, that is, at pressure ≥ 8 kbars (depth ≥ 25 km) in the upper mantle^{6,7}. Mantle-equilibrated pargasitic amphiboles are associated with these primary assemblages^{6,7}. Outside the primary augens the SPP peridotites contain recrystallized and secondary phases—pyroxene rims with an aluminium content lower than the cores, amphiboles of hornblende and tremolitic composition, and minor phases such as plagioclase, phlogopite, sodalite, apatite and scapolite^{6,7}. These mineral assemblages probably represent sub-solidus recrystallization and metasomatism that affected the peridotite body in the mantle and during ascent from the upper mantle.

To understand the origin of the SPP mantle body, I have compared its mineral chemistry, geothermometry and elemental/isotopic chemistry with those of mantle-derived peridotites from mid-ocean ridges on the one hand, and from continental or pre-oceanic rifts on the other. Previous studies have shown that peridotite bodies from pre-oceanic rifts¹¹ and passive

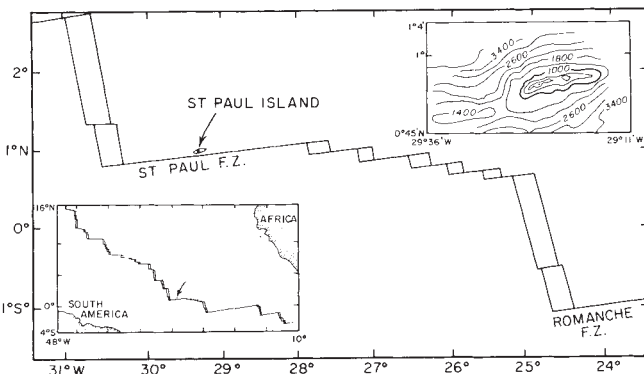


FIG. 1 Location of St Peter–Paul Island and of the massif (indicated by the 1-km isobath) that underlies the island. The geometry of the axis of the Mid-Atlantic Ridge and of its transform offsets (ref. 35 and J. G. Schilling, unpublished data) is shown schematically. Inset on upper right shows the morphology of the relief (in metres below sea level) the summit of which emerges at St Peter–Paul (adapted from ref. 8). Inset on lower left shows the location of St Peter–Paul in the equatorial Atlantic region.

margins¹² are systematically different in composition from peridotites recovered from mid-ocean ridges, probably reflecting a different upper-mantle composition and/or thermal structure in the different geotectonic environments¹³. To represent the uppermost mantle from mid-ocean ridges I have used a set of peridotites recovered by dredging, drilling and submersible from various sites along the axis of the northern and equatorial Mid-Atlantic Ridge (ref. 14 and E. Bonatti *et al.*, manuscript in preparation). The petrology and chemistry of these samples suggest a regionally heterogeneous, variably depleted upper mantle. The uppermost mantle from a continental pre-oceanic rift will be represented by the Zabargad (Red Sea) peridotite complex. The Zabargad peridotite body was emplaced in thinned continental crust, near the transition from a continental to an oceanic rift in the northern Red Sea¹¹. It consists of undepleted spinel lherzolite, similar in composition to pyrolite¹⁵, veined by metasomatized amphibole peridotite¹¹.

The chemistry of the primary mantle-equilibrated minerals, interpreted in terms of experimental work^{16,17}, can provide information on the degree of depletion and of partial melting undergone by the peridotite. The aluminium and sodium content of orthopyroxene and clinopyroxene decrease, and 100 Mg/(Mg + Fe²⁺) of orthopyroxene and 100 Cr/(Cr + Al) of spinel increase with increasing degree of depletion, reflecting the incompatible behaviour of Al, Fe and Na and the refractory behaviour of Mg and Cr during partial melting^{16,17}.

SPP SE9 and SE29 spinels are aluminium-rich⁷ relative to mid-ocean ridge peridotites (Fig. 2a), suggesting that these samples are relatively undepleted. As noted by Seyler and Mattson¹⁸, the SPP spinels fall outside the field of MAR peridotites in a plot of 100 Cr/(Cr + Al) against 100 Mg/(Mg + Fe²⁺), being enriched in Fe²⁺. In addition to being very aluminiferous, the spinels of the Zabargad lherzolites are also enriched in Fe²⁺ relative to mid-ocean ridge peridotites. This tendency of SPP and Zabargad spinels to be enriched in Fe²⁺ is observed also in sub-continental mantle peridotite massifs such as Balmuccia and External Ligurids^{18,19}. Iron-magnesium partitioning between olivine and spinel is strongly temperature-dependent^{20,21} so the displacement towards iron-rich composition of spinels from SPP, Zabargad and some sub-continental peridotites may reflect not only a different bulk composition but also a lower temperature of equilibration relative to oceanic peridotites.

In a plot of orthopyroxene Al₂O₃ against spinel 100 Cr/(Cr + Al) (Fig. 2b), which reflects the partitioning of aluminium between orthopyroxene and spinel, SPP SE9 and SE29 fall well outside the Atlantic peridotites array, as do the Zabargad spinel lherzolites. Experimental work^{22,23} suggests that the low partitioning of aluminium in orthopyroxene may reflect a lower temperature of equilibration and/or a different bulk composition of SPP and Zabargad peridotites relative to MAR peridotites¹³. The clinopyroxenes of SPP SE9 and SE29 peridotites are different in composition from those of MAR oceanic peridotites, but similar to those in Zabargad lherzolites, being highly enriched in sodium (Fig. 2c). These sodium-rich clinopyroxenes are characteristic of sub-continental mantle peridotites²⁴.

Pargasitic amphibole is part of the augen assemblage of SPP SE9 and SE29 peridotites, constituting one of the primary mantle-equilibrated phases^{6,7} in a situation similar to some Zabargad metasomatized amphibole peridotites¹¹. This contrasts with mid-ocean-ridge peridotites where primary mantle pargasite is absent, in general, although amphiboles produced by high-temperature secondary alteration are not uncommon²⁵.

Application of the Wells²⁶ two-pyroxene geothermometry provides an estimate of relative temperatures of equilibration of peridotites. Temperatures obtained for MAR peridotites are >900 °C, which is higher than the temperature of equilibration estimated for the Zabargad lherzolites. This difference probably reflects a cooler upper mantle beneath continental rifts than mid-ocean ridges¹³. Estimated temperatures for SPP SE9 and

SE 29 are <900 °C, that is, lower than the range of MAR peridotites but close to the temperatures estimated for the Zabargad lherzolites. This may imply that SPP was never caught in upwelling hot mantle beneath a mid-ocean ridge, which would have resulted in a higher degree of melting and a higher equilibration temperature, as shown by MAR peridotites. Independent support for temperatures of equilibration of SPP and Zabargad lower than those of MAR peridotites is provided by spinel Mg/Fe²⁺ (Fig. 2a) and orthopyroxene/spinel Al₂O₃ (Fig. 2b) data, as discussed above.

The chondrite-normalized rare-earth element (REE) distribution of SPP peridotites (Fig. 3) displays a strong light-REE enrichment^{7,27}, which is not shown by Atlantic oceanic peridotites²⁸. This light-REE enrichment is similar to that observed in Zabargad metasomatized amphibole peridotites, which has been attributed to the injection of light-REE- and H₂O-rich fluids during one or several metasomatic events in the mantle¹¹. Such metasomatism is not generally observed in mid-ocean ridge peridotites but is inferred to operate in continental rifts as in the northern Red Sea^{11,29} or in the East African rift^{30,31}.

The Nd and Sr isotopic composition of SPP peridotites are similar to Zabargad metasomatized amphibole peridotite (ref. 29 and H. Brueckner *et al.*, unpublished data) to the inferred mantle source of East African rift basalts³⁰, and to ocean island basalt, rather than to mid-ocean-ridge basalt⁷. The nepheline-normative alkali basalt associated with the SPP massif is very

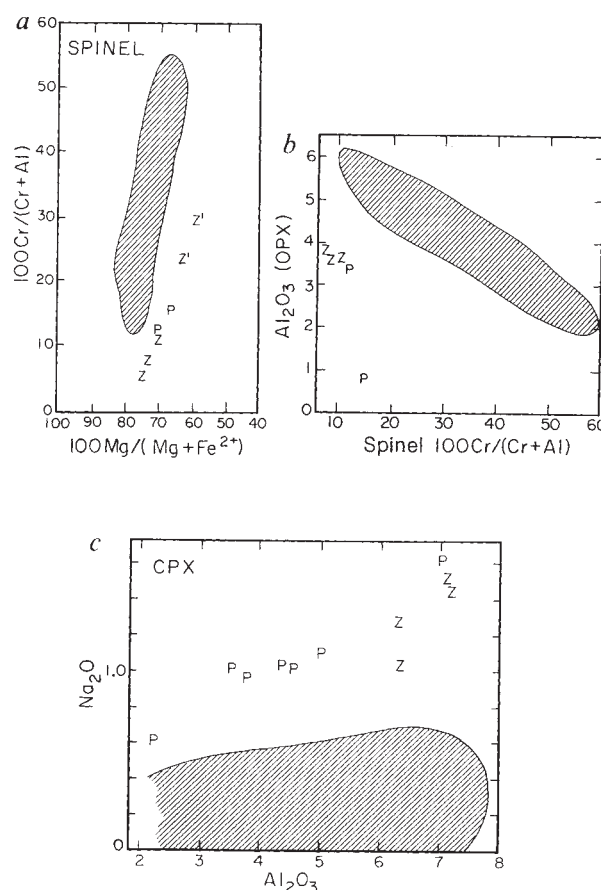


FIG. 2 a, 100 Cr/(Cr + Al) plotted against 100 Mg/(Mg + Fe²⁺) for spinels from St Peter-Paul SE9 and SE29 peridotites⁷, denoted P, and Zabargad spinel lherzolites and plagioclase peridotite¹¹, denoted Z and Z', respectively. Field of oceanic peridotites^{14,21}, hatched area is also shown. b, Orthopyroxene Al₂O₃ plotted against spinel 100 Cr/(Cr + Al) of St Peter-Paul SE9 and SE29 peridotites⁷ and Zabargad spinel lherzolites¹¹ compared to Mid-Atlantic Ridge peridotites (ref. 14 and E. Bonatti *et al.*, unpublished data). c, Al₂O₃ plotted against Na₂O in clinopyroxenes from St Peter-Paul peridotites^{6,7} and Zabargad spinel lherzolites¹⁰ compared to peridotites from the Mid-Atlantic Ridge (ref. 14 and E. Bonatti *et al.*, unpublished data).

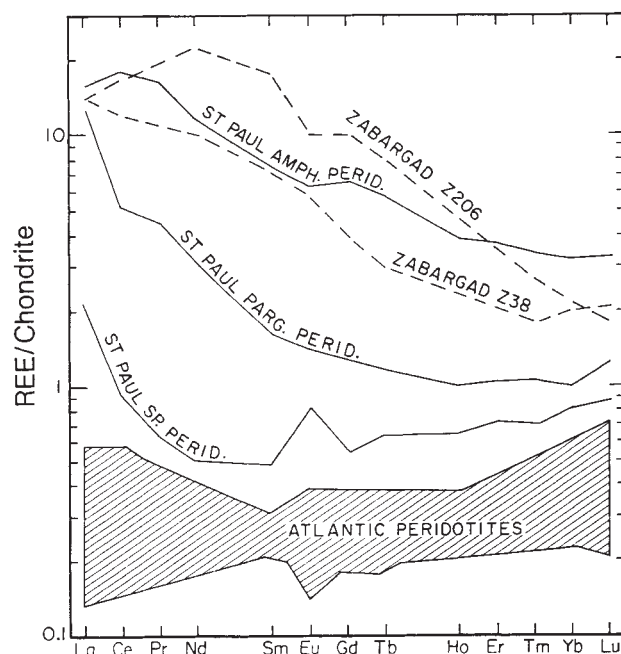


FIG. 3 Chondrite-normalized rare-earth element distribution of St Peter–Paul spinel, pargasite and amphibole peridotites⁷ compared to Zabargad amphibole peridotites¹¹ and to peridotites from the equatorial Atlantic (E. Bonatti, J. L. Joron and G. Ottonello, unpublished data).

different in chemistry from mid-ocean-ridge basalts but is isotopically identical to the SPP peridotites^{7,9,10}. Roden *et al.*⁷ ascribed the light-REE and other large-ion-lithophile elements enrichment of SPP peridotites to a metasomatic event that affected the mantle. Nd isotope data suggest that this event occurred ~155 Myr ago⁷.

I conclude that SPP exposes not sub-mid-ocean-ridge mantle, but a body with the properties of mantle from a continental pre-oceanic rift. SPP could be a relict fragment of pre-Atlantic-rift lithospheric upper mantle, left behind during the opening of the equatorial Atlantic. This hypothesis is consistent with the probable age of ~155 Myr for the metasomatic event that affected SPP⁷, because the equatorial Atlantic was probably in a pre-oceanic continental-rift stage at that time.

The mechanism by which the SPP mantle body could have been left behind during the opening of the Atlantic, and now be within the transform offset of two segments of the Mid-Atlantic Ridge, might be related to SPP being located in a large megashear zone, which crosses the equatorial region from coast to coast. This region is characterized by very short disrupted spreading segments offset by several very large and a number of smaller transforms. Sea-floor spreading in this morphotectonic environment is likely to be sluggish and processes such as transform migration, 'oscillatory spreading'³² and transform-related vertical tectonics³³ might contribute to the possibility that relicts of pre-oceanic sub-continental-rift lithosphere are left behind during the opening. The abundance in the SPP mantle body of hydrated phases such as amphiboles and phlogopite might have contributed to its uplift by lowering its density.

Fertile, water-rich and large-ion-lithophile-rich mantle similar to SPP would provide a suitable source for ocean-island basalts⁷ and could cause the enhanced melting inferred in some hotspot areas, such as that at 34°–45° N in the Mid-Atlantic Ridge without requiring abnormally high mantle temperatures (ref. 34 and E. Bonatti, manuscript in preparation). □

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Articulated halkieriids from the Lower Cambrian of north Greenland

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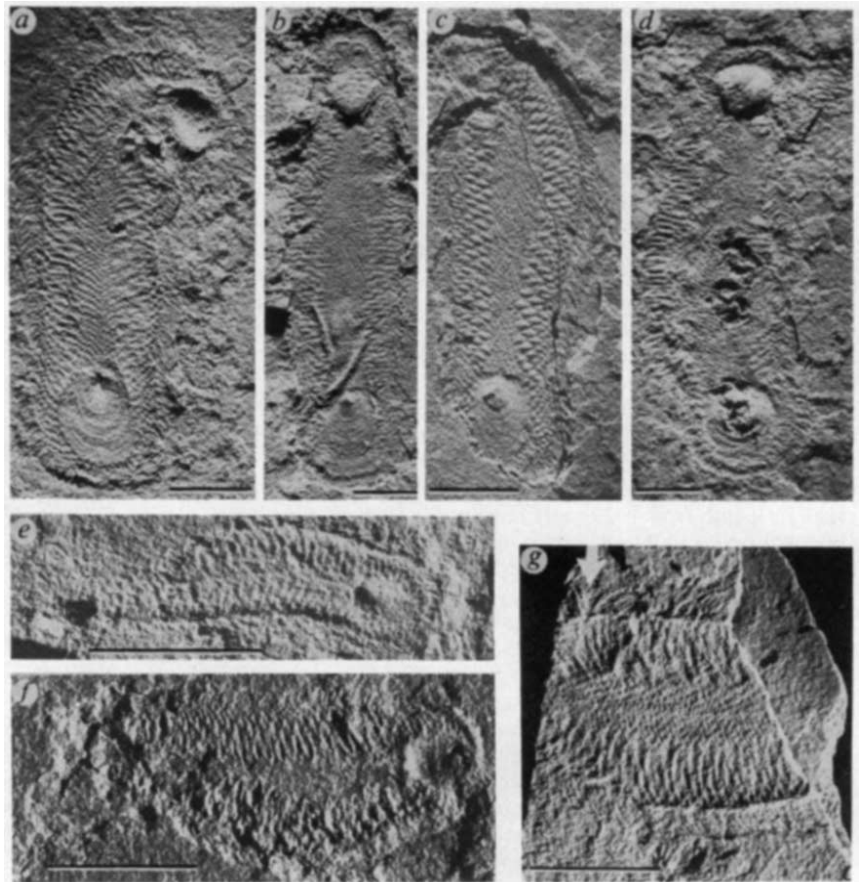
METAZOAN skeletons appear abruptly in the fossil record near the base of the Cambrian (~540 Myr BP (before present))^{1,2}. In the initial stages of diversification, familiar skeletal remains such as sponge spicules, brachiopod valves and echinoderm ossicles are outnumbered by a remarkable array of skeletal types, many of millimetric size^{3–9}. Particularly conspicuous in these early assemblages are various sclerites, some of which are inferred to have formed cataphract coverings of slug-like metazoans. Recognition of articulatory facets and fused sclerites contribute to partial scleritome reconstructions^{8–11}, but discovery of articulated material is a vital ingredient in understanding the palaeobiology of these extinct groups. Here we report complete halkieriid scleritomes from the Lower Cambrian of north Greenland. This finding broadly confirms the predictions of an earlier hypothetical reconstruction¹¹, but reveals entirely unexpected anterior and posterior shells. It supports also the proposed phylogenetic link between the halkieriids and *Wiwaxia corrugata*, a metazoan with a cataphract scleritome best known from the Middle Cambrian Burgess Shale.

In July 1989 a four-man expedition to a locality near J. P. Koch Fjord, Peary Land, collected more than 3,000 soft-bodied specimens from talus, shed from a ~5-m-thick unit of fissile shale of the Buen Formation¹². The stratigraphic horizon of these beds within the Buen Formation is still unresolved, in part because the local contact with the adjacent and underlying dolomites of the Portfeld Formation is clearly faulted. The overall age of the Buen within the Lower Cambrian also remains

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FIG. 1 Articulated halkieriids from the Lower Cambrian Buen Formation, Peary Land, northern Greenland. *a*, Entire specimen with anterior end recurved to right (Geological Museum, Copenhagen specimen no. MGUH 19728). *b*, Entire specimen. Note trace fossils impressed onto posterior region (MGUH 19729). *c*, Entire specimen with anterior recurved to left. Parts of anterior and posterior covered with sediment (MGUH 19730). *d*, Entire specimen. Hollows in centre of body may represent alimentary canal (MGUH 19731). *e*, Entire specimen, twisted over near anterior end (left) to show opposite side of body (MGUH 19732). *f*, Entire specimen (MGUH 19733). *g*, Portion of mid-length of body (MGUH 19734). Arrow points to bunched arrangement of siculate sclerites. All specimens are preserved in dorsoventral attitude, although *e* is twisted over. Specimens seem to represent decalcified compressions and are preserved in either positive (*a-d*) or negative (*e-g*) relief. All specimens from Geological Survey of Greenland sample 340103, deposited in the type collection, Geological Museum, Copenhagen (MGUH). All specimens except *f* coated with sublimate of ammonium chloride. Scale bars, 10 mm.



uncertain. It seems, however, that the fauna comes from near the base of the formation, rather than close to the top as supposed¹³. The age could, therefore, be as old as mid-Early Cambrian (early Atdabanian).

Our collecting confirms the initial report of this exceptional fauna¹³, with the assemblage dominated by lightly skeletalized arthropods. New records include Burgess Shale-type¹⁴ poly-

chaete and priapulid worms, sponges, palaeoscolecids (a group of worms with distinct papillate annuli^{15,16}), simple trace fossils, and most significantly, articulated halkieriids (Figs 1 and 2).

Until now halkieriids have been known almost entirely from isolated sclerites, typically replaced by diagenetic phosphate and isolated by acid digestion in the laboratory^{3,9,11}. Despite

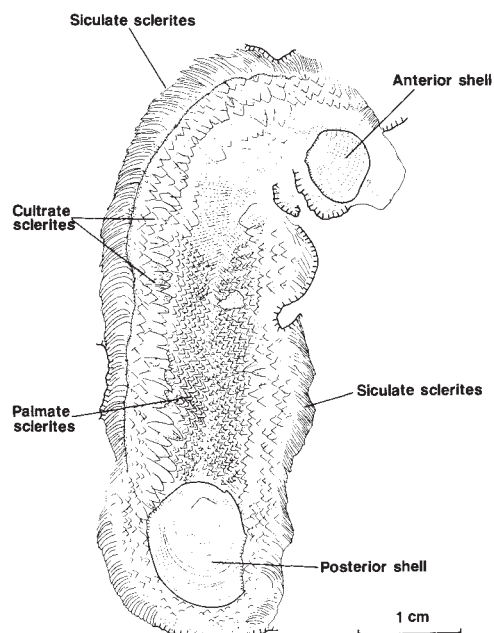


FIG. 2 Diagrammatic sketch of articulated halkieriid (MGUH 19728); compare with Fig. 1*a*. The drawing is interpretative, and not designed to show the precise position of each sclerite. The anterior is recurved to the right and some details of this region are less clear, including the anterior shell. The posterior shell is also partially incomplete, but the overall distribution of siculate, cultrate and palmate sclerites is clear. In this specimen the cultrates, equivalent to the upper laterals of *Wiwaxia* are clear, but those equivalent to the lower laterals (see Fig. 3*b*) are obscure.

wide morphological variability, at least three basic types of sclerite have been recognized. These are siculates (crescentic, asymmetrical), cultrates (relatively elongate, approximately symmetrical), and palmates (flattened, somewhat asymmetrical). Reconstruction of the scleritome is made difficult because only very seldom are a few sclerites found articulated (ref. 17; A. B. Fedorov, personal communication), so the mutual relations of the various types of sclerite have remained speculative. An important clue comes from articulated specimens of a descendant form, *Wiwaxia corrugata* from the Middle Cambrian Burgess Shale of British Columbia¹⁸. Although differing in various respects, the scleritome of *Wiwaxia* has been taken as a basis for halkieriid reconstructions^{9,11}. The discovery of the north Greenland material now allows this hypothesis to be tested.

Twenty-one articulated specimens are known. All seem to belong to a single species, most probably assignable to *Halkieria* which is widely distributed in the Lower Cambrian^{4,5,9,11}. Of the available specimens, eight are more or less complete and range in length from ~29 to 71 mm (mean 50 mm). Specimens (Figs 1a, c, d and 2) are variously curved, indicating that the body was originally flexible. In transverse section the body was probably relatively compressed.

Several distinct zones of sclerites are recognizable; these correspond closely to those identified in *Wiwaxia*¹⁸. The margins were rimmed by a file of siculate sclerites (Figs 1a, c, 2 and 3c). Although crescentic, the precise morphology of individual sclerites is difficult to resolve because of their close packing. In some areas a group of siculates seems to originate from a common area, giving a bunch-like arrangement (Fig. 1g). The zone of siculates is delimited from the remainder of the body by a groove. The adaxial zone is occupied by lateral (cultrate)^{9,11} sclerites. What may correspond to the lower lateral sclerites of *Wiwaxia*¹⁸ are obscurely preserved, but seem to consist of lanceolate sclerites disposed parallel to the antero-posterior axis (Fig. 3b). The equivalents to the upper lateral sclerites, however, are clearly displayed. They consist of several rows that are

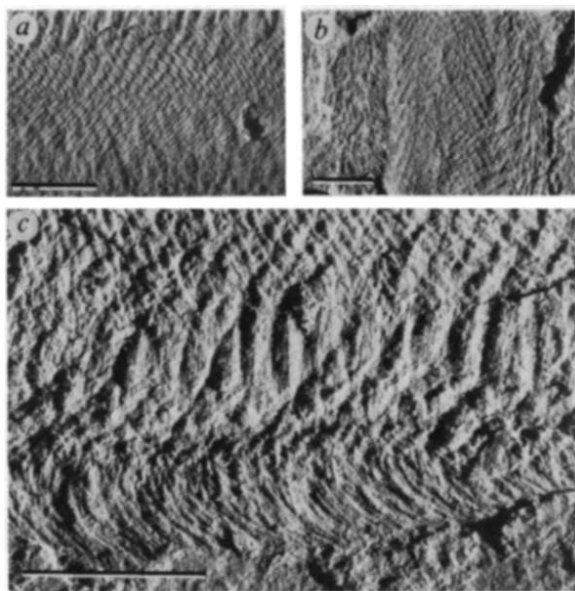


FIG. 3 Detail of sclerite arrangement in articulated halkieriids. a, Palmate and cultrate sclerites (equivalent to upper lateral sclerites of *Wiwaxia*) (MGUH 19728). b, Palmate and siculate sclerites. Note margins of body have been folded beneath rest of halkieriid so direction of siculates is reversed, flanked by apparently cultrate sclerites (equivalent to lower lateral sclerites of *Wiwaxia*) (MGUH 19729). c, Palmate, cultrate (equivalent to upper lateral sclerites of *Wiwaxia*) and siculate sclerites (MGUH 19728). Scale bars, 5 mm.

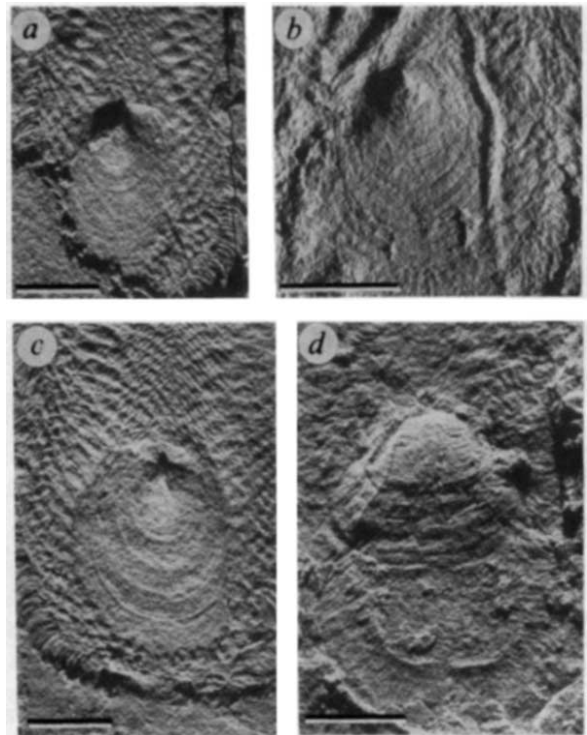


FIG. 4 Detail of posterior (a-c) and anterior (b) shells. a, MGUH 19730; b, MGUH 19729; c, MGUH 19728; d, MGUH 19729. Note apparent retraction of shell from anterior-most region (bottom of photograph), where marginal array of sclerites surrounds approximately quadrate smoother area. Scale bars, 5 mm.

directed adaxially, increase in size in that direction, and overlap the central zone of palmate sclerites (Figs 1a, c, f, 2 and 3c). The palmates consist of regular imbricated rows that run obliquely-posterior towards the midline, the latter defined by a median groove (Figs 2 and 3a, b). The dorsal region also bears a regular series of rib-like structures (Fig. 2) running at right angles to the direction of the palmate rows. These might represent cuticular ridges or skeletal elements deeper in the body. In addition to conforming to the shape of isolated halkieriid sclerites, the cultrates and palmates also show the characteristic ribbing. Original composition is uncertain, but the relative relief of the sclerites indicates once mineralized walls, presumably of calcium carbonate^{9,11}.

In addition to the sclerites, which in an average-sized specimen may have totalled ~2,000, there is a prominent shell at either end of the body. The anterior shell is somewhat rectangular in outline with blunt spur-like extensions on either side (Fig. 4d). It is relatively convex. The oval posterior shell (Figs 2 and 4a-c) superficially resembles some valves of monoplacophorans and inarticulate brachiopods. It is less convex than the anterior valve, but has a prominent apex. There is some evidence that the anterior margin of this shell was shallowly arched. Both shells have co-marginal growth lines (Fig. 4), and also an ornamentation of fine striations that run obliquely to the growth increments.

Little is known of the soft parts, but the siculates presumably delimited a soft ventral sole. Irregular linear hollows (Fig. 1d) in some specimens may represent the alimentary canal, but a feeding apparatus comparable to that of *Wiwaxia*¹⁸ has not so far been identified. If present, this apparatus may be concealed beneath the anterior shell.

The discovery of articulated halkieriids is significant for several reasons. It confirms the basic structure of the halkieriid-wiwaxiid body plan, and suggests that comparisons with groups

such as aplacophoran molluscs or chrysopetalid annelids may be invalid. It is also clear that in contradistinction to the hypothesis of the halkieriid scleritome¹¹ the palmates were dorsal and cultrates were lateral in position (a point already addressed in ref. 9). The articulated specimens differ from *Wiwaxia* in several respects. Although similar in size to *Wiwaxia*¹⁸, this halkieriid has many more sclerites that are proportionally smaller. The Greenland halkieriids lack dorso-lateral spines and have a greater length-to-width ratio. The presence of anterior and posterior shells (Figs 2 and 4), however, is entirely unexpected. In the Lower Cambrian assemblages of small skeletal fossils there is a wide variety of breviconic shells, whose affinities for the most part have remained enigmatic. Although none of the published material seems to be identical to either the anterior or posterior shell of the Greenland material, we believe that many such isolated shells will be shown to derive from scleritomes of either halkieriids or near relatives within the Coeloscleritophora¹⁷.

This discovery also re-opens the problems of how halkieriids grew. Sclerites evidently were secreted at a fixed size, so that growth was probably achieved by either interpolation of new sclerites or moulting. Whereas evidence for the latter exists in *Wiwaxia*¹⁸, in the Greenland halkieriids growth seems to have been accommodated by sclerite interpolation and marginal accretion of the shells. Recent suggestions, however, that halkieriids are related to other sclerite-bearing Palaeozoic worms such as tommotiids (at present known only from two types of phosphatic sclerite that presumably armoured a worm-like organism) and machaeridians^{19,20} (a worm-like group with several files of calcareous plates secreted by accretionary growth) remain speculative. Transitions between scleritomes of machaeridians and either halkieriids¹⁹ or tommotiids²⁰⁻²² are conceivable despite differences in style of sclerite growth²³ and mineralogy respectively.

Discovery of exceptionally preserved Lower Cambrian fossils such as articulated halkieriids or sclerites of the hitherto enigmatic phosphatic *Microdictyon* in association with soft parts from the Chengjiang lagerstätte in South China²⁴ is of general importance²⁵. It makes palaeobiological assessments far more secure than is possible using isolated sclerites, and also brings taxonomic order to an area where, until recently^{3,6,8,9}, form taxonomy applied to different sclerite shapes has been rampant. The recognition of anterior and posterior shells reinforces the likelihood that some Cambrian scleritomes are more complex than hitherto realized. Their accretionary mode of growth suggests that although halkieriids are a distinct group, a relationship to the molluscs¹⁸ remains a strong possibility. In conclusion, such articulated scleritomes will do much to unravel the phylogenetic affinities of many of the Cambrian problematical forms. □

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Directly measured rapid growth of a deep-sea barnacle

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ESTIMATES of the growth rates of organisms in the deep sea have usually been indirect owing to the remote and hostile nature of this environment. Consequently, it is not clear whether slow growth rates are the general rule¹ or the exception²⁻⁴. I have now directly measured the growth rate of a barnacle on the deep-sea floor. To my knowledge this is the first direct measurement of the growth of a deep-sea specimen in its natural habitat: previous direct measurements have been unrepresentative, coming from hydrothermal vents. Measurements of the length of the barnacle over a period of seven months demonstrate a high growth rate: the body length increased from less than ~1 mm to 10 mm in only six months, and the specimen probably reached sexual maturity in about seven months. This rate seems to be greatly enhanced by the annual deposition of phytodetrital material.

The time-lapse camera system Bathysnap⁵ was used in November 1986 at a depth of 1,526 m on the Goban Spur 200 km southwest of the UK to photograph 2 m² of seabed every 4 hours. A barnacle cyprid soon settled on a plastic current-direction vane in the field of view and grew there over the next 228 days (Fig. 1). The instrument, recovered on 19 September 1987, was extensively colonized by many specimens of the barnacle *Poecilasma kaempferi*. The photographed specimen, although not recovered, was indistinguishable from these and was assumed to be of the same species.

Growth rates usually decrease with increasing age, and there are a variety of mathematical descriptions of this relationship. One of the most frequently used is the Von Bertalanffy growth curve

$$L_t = L_{\infty} - (L_{\infty} - L_0) e^{-K(t-t_0)}$$

where L_t is size at time t , L_{∞} is the maximum size attainable,

TABLE 1 Growth rate data

	Estimate	Standards error	95% confidence intervals
All data			
R^2	410.8		
K	0.00350	0.000110	0.00328 to 0.00372
L_0	-0.4261	0.27016	-0.9589 to 0.10674
T_0	-0.2407	0	
Pre-deposition			
R^2	44.4		
K	0.00218	0.000081	0.00201 to 0.00234
L_0	1.0679	0.1338	0.8029 to 1.3329
T_0	-1.074	0	
Post-deposition			
R^2	100.7		
K	0.00946	0.000592	0.00828 to 0.01063
L_0	0.6943	0.7961	-0.8897 to 2.278
T_0	116.699	0	

FIG. 1 Examples of photographs of the current-direction vane at a depth of 1,526 m. The location of the growing barnacle is indicated. The dimensions of the vane are 72×151 mm.

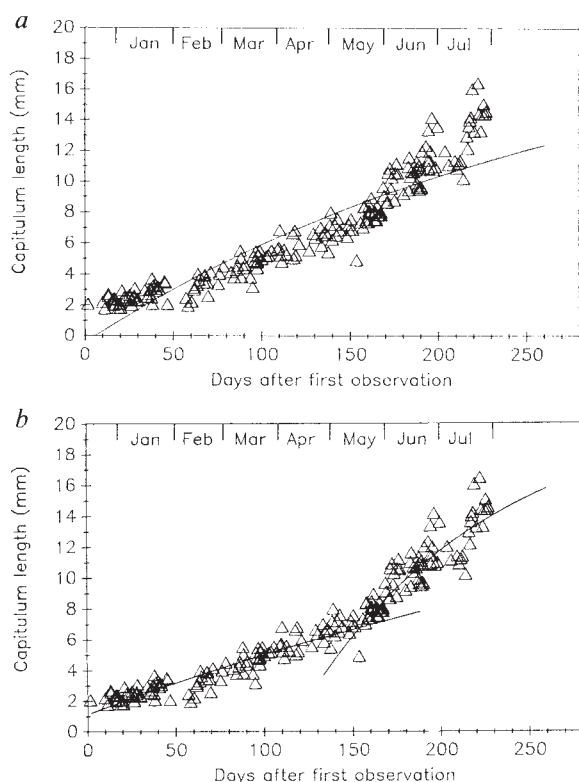
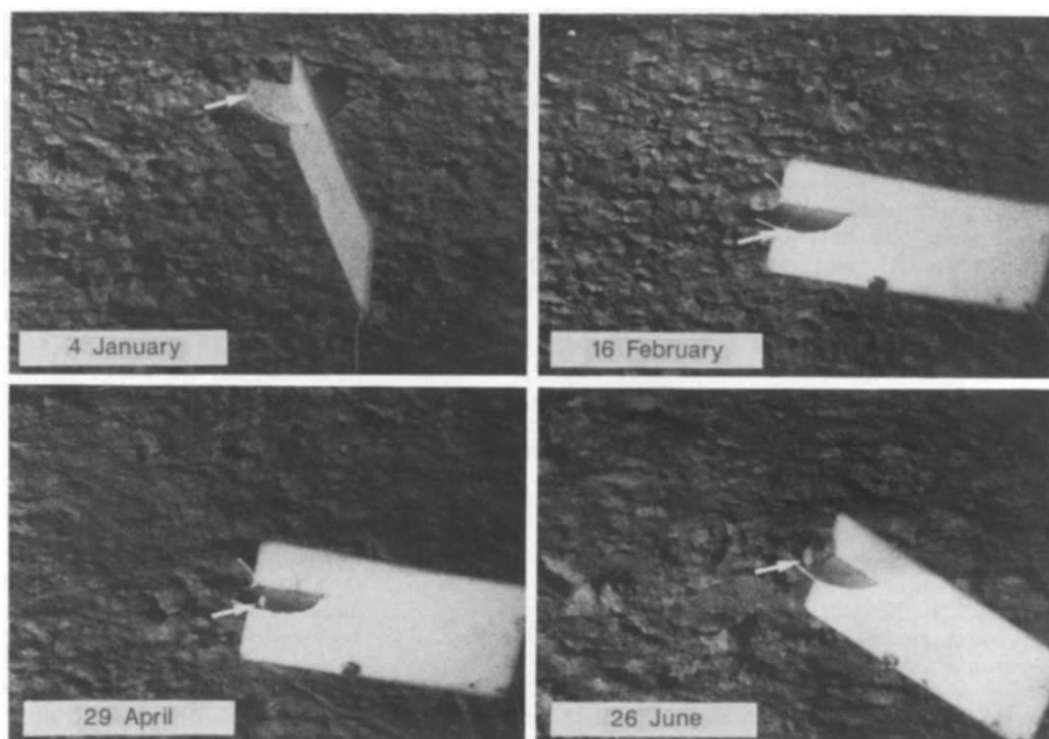


FIG. 2 The measured length of the barnacle capitulum during the period of observation. The curve in *a* is the best fit for the entire data-set giving a residual standard deviation $S_{y/x} = 2.10$ mm, whereas in *b*, curves have been fitted to lengths before and after 15 May (when deposition of phytodetritus began), giving $S_{y/x} = 0.397$ and 1.244 mm respectively, a clear improvement in the fit.

L_0 is the settlement length, t_0 is the settlement time and K is the rate constant⁶. Using a nonlinear least-squares procedure⁷, this curve was fitted to the data to facilitate comparisons with published growth data (Fig. 2a). A value of 21 mm was taken for L_∞ , this being the largest reported size for this species⁸ (R. Williams, personal communication). The rate constant derived was $3.5 \times 10^{-3} \text{ d}^{-1}$ (1.28 yr^{-1}), a value several times higher than those previously calculated for deep-sea megafaunal invertebrates, and comparable to values calculated for shallow-water fauna. However, barnacles typically have high growth rates, and the values calculated here are several times lower than rates reported for near-surface barnacles⁹.

The distribution of the residuals in Fig. 2a is clearly not uniform, indicating that the data do not conform well to this model. Indeed there seems to be an increase in growth rate in mid-May. As has been described previously in this region^{10,11}, between 15–20 May there was a substantial deposition of detrital material derived from the spring phytoplankton bloom in the surface waters. This material, which covered a high proportion of the seabed, forms a highly mobile layer which is readily put into suspension by the near-bed currents¹¹. As barnacles are suspension feeders, deposition of such material is likely to enhance their food supply substantially.

Two growth curves have been fitted to the data for the periods before and after the deposition of detritus (Fig. 2b), resulting in a better fit to the growth model. (Note the decrease in the value of the residual sum of squares, R^2 ; Table 1.) A sharp increase in growth rate is implied by these curves, with the growth constant increasing by a factor of 4.4 in mid-May. Whichever of the three growth curves in Fig. 2 is accepted, the rate is particularly fast. Knowledge of *Poecilasma kaempferi* is limited to specimens collected as epizoids of the lithodid crab *Neolithodes grimaldi*, and it has been inferred that the species grows rapidly in order to reach maturity before the host moults⁸.

Previous direct studies on growth in the deep sea have used mark-and-recapture techniques and have been on hydrothermal vent molluscs¹². Rates of growth were much higher (K up to 0.096 yr^{-1}) than rates derived in non-vent environments using radiochemical techniques¹, or implied from recolonization

studies¹³. However, other indirect growth studies performed in typical deep-sea habitats indicate that many invertebrate megabenthos grow at rates similar to those in the vents and not much lower than those in shallow water^{14–18}.

Several small deep-sea species have high rates of growth and early maturation when presented with an appropriate food source² or vacant habitat^{19,20} and some respond rapidly to natural deposition of food^{21,22}. However, influences on the megafauna have previously been inferred only from the annual periodicity in the reproductive cycles of a few species¹⁹. The present data provide direct evidence that at least one megafaunal species grows rapidly in its natural habitat and can respond quickly to elevated food input by increasing its growth rate several times. □

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Postsynaptic NMDA receptor-mediated calcium accumulation in hippocampal CA1 pyramidal cell dendrites

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IN the CA1 hippocampal region, intracellular calcium is a putative second messenger^{1,2} for the induction of long-term potentiation (LTP), a persistent increase of synaptic transmission produced by high frequency afferent fibre stimulation³. Because LTP in this region is blocked by the NMDA (N-methyl-D-aspartate) receptor antagonist AP5 (DL-2-amino-5-phosphonovaleic acid) (ref. 4) and the calcium permeability of NMDA receptors is controlled by a voltage-dependent magnesium block^{5,6}, a model has emerged that suggests that the calcium permeability of NMDA receptor-coupled ion channels^{7–10} is the biophysical basis for LTP induction^{11–15}. We have performed microfluorometric measurements¹⁶ in individual CA1 pyramidal cells during stimulus trains that induce LTP. In addition to a widespread component of postsynaptic calcium accumulation previously described¹⁷, we now report that brief high frequency stimulus trains produce a transient component spatially localized to dendritic areas near activated afferents. This localized component is blocked by the NMDA receptor antagonist

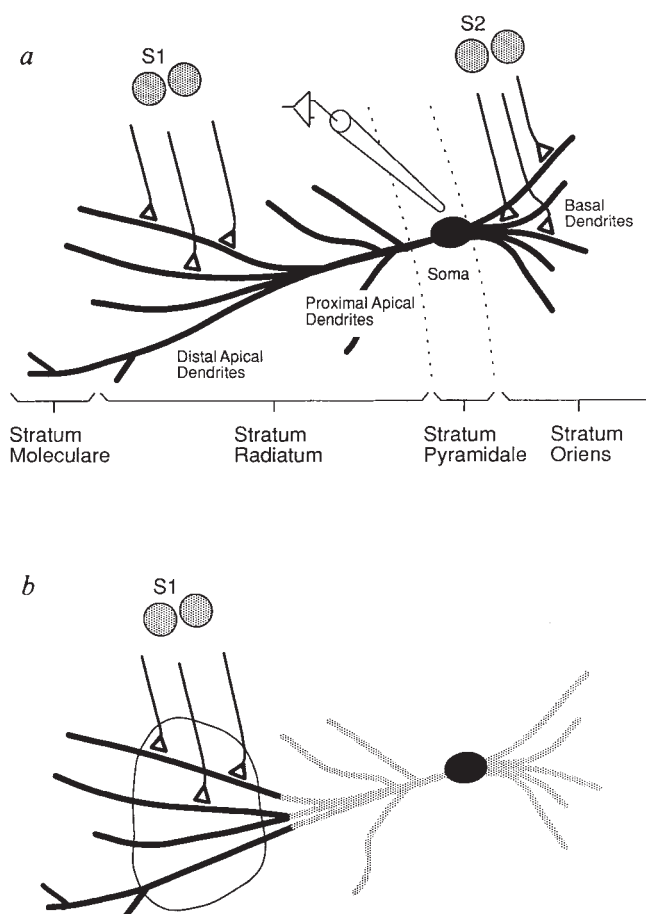


FIG. 1 Cell-electrode geometry. *a*, Stimulus electrodes positioned in stratum radiatum (electrode S1; distal apical dendrites) or stratum oriens (electrode S2; basal dendrites) activated a group of afferent fibres with average orientation parallel to the cell body layer. An extracellular electrode near the cell soma recorded the population spike. *b*, AP5-insensitive calcium accumulations previously observed at moderate frequency synaptic stimulation¹⁷ are indicated by the patterned region. In most experiments the stimulating electrode was placed 300–500 μm from the cell in distal stratum radiatum (position S1), activating fibres synapsing on the circled region (distal apical dendrites). This distal position facilitates measurements of calcium accumulations with minimal interference from AP5-insensitive accumulations. The cell diagram is a coarse sketch from a fluorescence image of the cell in Fig. 2.

METHODS. Slice preparation and maintenance followed standard procedures³⁰. Electrophysiological and imaging techniques were similar to those previously described¹⁷. Individual pyramidal cells were iontophoretically filled with fura-2 (–1 nA, 5 min). The electrode was removed and 30–60 min allowed for the dye to diffuse to distal dendrites. From comparisons of cell soma fluorescence with that of calibration solutions, intracellular fura-2 concentrations were estimated to be 50–150 μM . Cells with higher fura-2 levels showed diminished AP5-sensitive accumulations, and the time courses of all accumulations were slower. An extracellular electrode recorded the population spike evoked by 0.1 Hz test pulses. For all cells used in the imaging experiments reported here, nearby field potentials displayed LTP (at least a 50% increase in the amplitude of the population spike that reached a stable baseline 10 min after high frequency stimulation). Stimulus trains of 100 Hz for 0.8–1.0 s produced a persistent increase in amplitude of the population spike, from a prestimulation value of $320 \pm 180 \mu\text{V}$ to $810 \pm 370 \mu\text{V}$ ($n=20$) for stratum radiatum stimulation and from an average of $250 \mu\text{V}$ to $710 \mu\text{V}$ ($n=4$) for stratum oriens stimulation. Field-potential measurements of presynaptic volley amplitude made at 50 μm intervals along the length of imaged cells showed that activated fibres were generally confined to dendritic regions (Fig. 1), with presynaptic volley amplitude evoked by stratum radiatum stimulation typically 20 times larger in distal stratum radiatum than in stratum oriens. Experiments conducted with AP5-containing saline used 100 μM DL-AP5 (Sigma) or 50–100 μM D-AP5 (Sigma) with 25–30 min for washing out.

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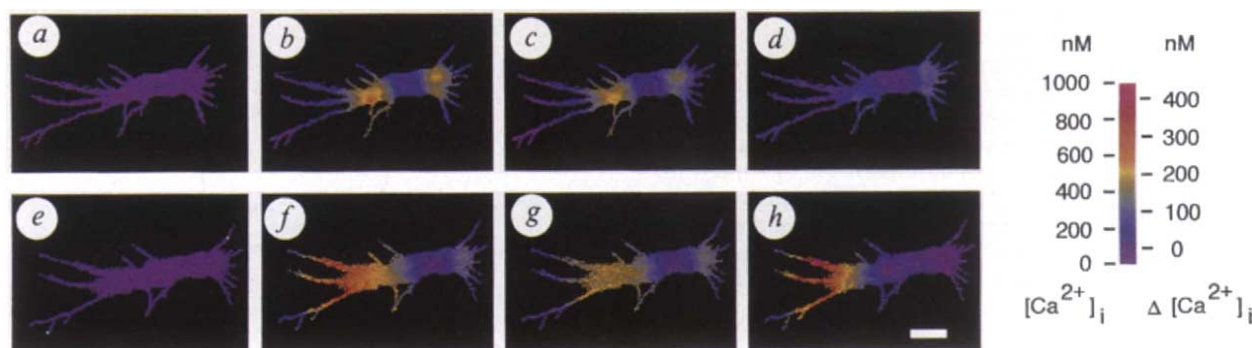


FIG. 2 High-frequency stimulations produced AP5-sensitive accumulation in dendritic regions postsynaptic to activated afferents. A stimulating electrode in position S1 (Fig. 1) delivered stimulus trains of 100 Hz (1 s) or 20 Hz (5 s) in control- and AP5-containing saline. Calcium levels were computed from ratios of 340 nm and 380 nm fluorescence images. The camera lucida-like masks that define pixels where the calcium concentration is displayed in pseudocolour were produced from 380 nm excited fluorescence intensity profiles. Slight differences in the masks result from slight differences in the focus position for the two experiments. *a, e*, Resting calcium distribution in the absence of stimulation. *b, f*, Calcium accumulation in the proximal apical and basal dendrites produced by 20 Hz stimulation of afferent fibres (map computed from images taken from $t=4-5$ sec from the start of the stimulation) in control saline, and *c, g*, in the presence of AP5. *d, h*, Image produced by pixel-by-pixel subtraction of distribution in AP5-containing saline (*g*) from the corresponding distribution in control saline (*f*) shows a large AP5-sensitive accumulation during the tetanus in the area directly postsynaptic to the activated afferent fibres. The scale to the left of the colour bar refers to: *a-c, e-g*, and the scale to the right of the colour bar refers to *d* and *h*. Scale bars, 100 μm.

start of the stimulation). *g*, Distribution under identical conditions as *f*, but in the presence of AP5. *h*, Image produced by pixel-by-pixel subtraction of distribution in AP5-containing saline (*g*) from the corresponding distribution in control saline (*f*) shows a large AP5-sensitive accumulation during the tetanus in the area directly postsynaptic to the activated afferent fibres. The scale to the left of the colour bar refers to: *a-c, e-g*, and the scale to the right of the colour bar refers to *d* and *h*. Scale bars, 100 μm.

METHODS. In experiments that compared accumulations in AP5-containing and control saline, protocols similar to those previously described³¹ were used to eliminate the complication of changes in accumulation caused by the potentiation: (1) cells were stimulated and measured in AP5 followed by a second stimulation/measurement sequence in control saline; (2) the stimulated pathway was fully pre-potentiated by pre-imaging stimulus trains. In some experiments cells were tested in control saline followed by AP5-containing saline without explicit prepotentiation with no qualitative differences in results, suggesting that the potentiation of transmission only marginally affects the calcium accumulations under the stimulus conditions we used.

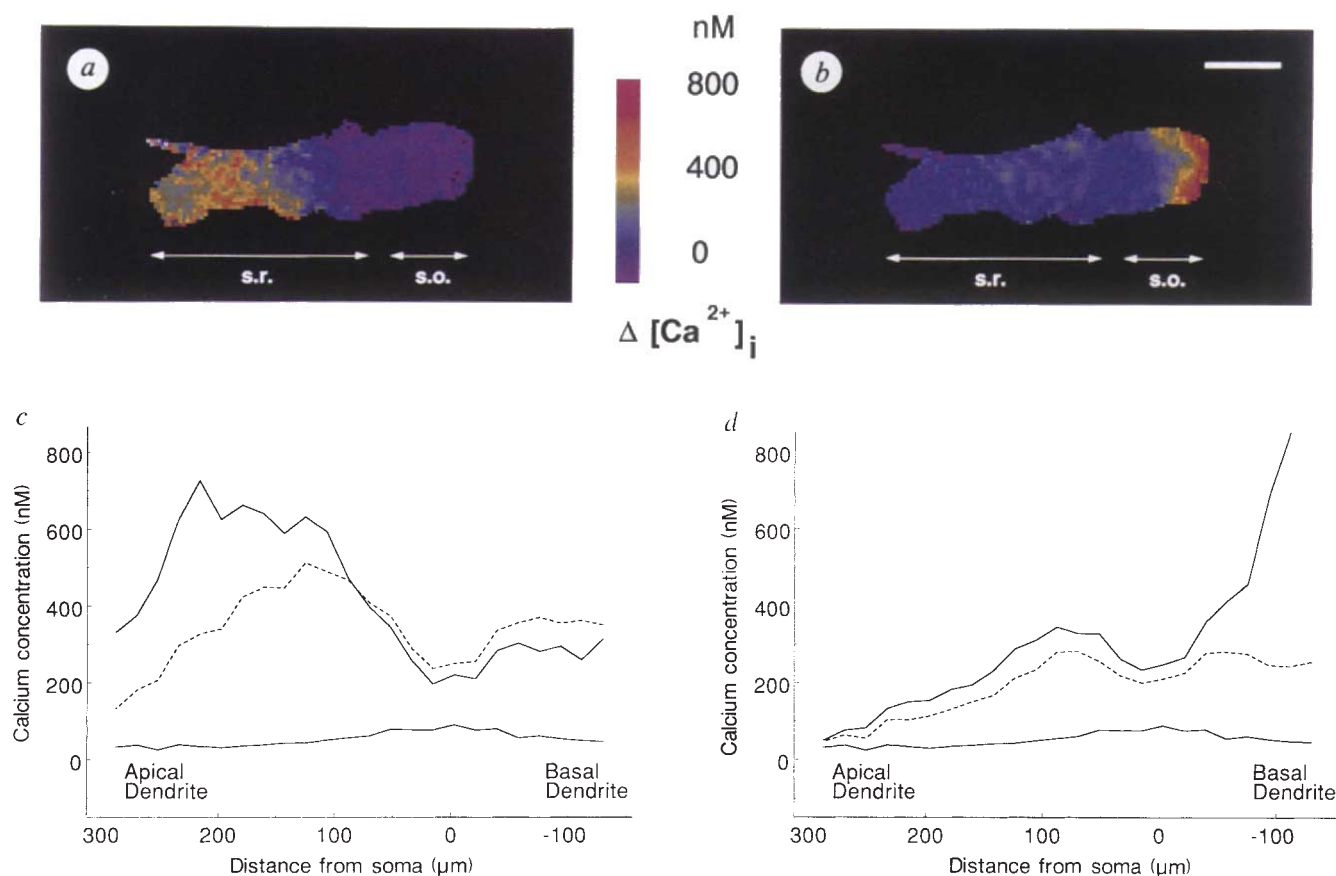


FIG. 3 AP5-sensitive accumulations correspond to terminal fields of stimulated afferent fibres. *a, b*, Difference maps (produced as in Figs 1*d* and *h*) representing the AP5-sensitive component of calcium accumulation produced by 100 Hz, 1 s stimulation of afferents in *a*, distal stratum radiatum and *b*, stratum oriens. *c, d*, Calcium accumulation in dendritic regions plotted against distance from the cell soma centre in the presence of AP5 (dashed

lines) and in control saline (solid line), with resting calcium levels also plotted (lowest solid line); *c*, stratum radiatum stimulation; *d*, stratum oriens stimulation. The difference between the line plot for control saline and the corresponding plot for AP5-containing saline represents the AP5-sensitive component shown for all cell locations in the two-dimensional maps in Fig. 3*a, b*. Scale bars, 100 μm.

AP5. The results directly confirm the calcium rise predicted by NMDA receptor models of LTP induction.

Optical measurements of intracellular free calcium were performed on pyramidal cells in region CA1 of guinea pig hippocampal slices microinjected with the fluorescent calcium indicator fura-2 (ref. 18). A small group of afferent fibres was activated by electrical stimulation with a small bipolar electrode positioned in distal regions of stratum radiatum, 200–350 μm from the cell-body layer (S1; Fig. 1a). Because activated fibres run predominantly parallel to the cell-body layer (stratum pyramidale) and diverge only several hundred micrometres over the electrode–cell distances used in our experiments¹⁹, calcium accumulation produced by influx through postsynaptic calcium-permeable channels near the presynaptic terminals of the activated afferents should be observed in distal apical dendritic regions (circled in Fig. 1b). It has been demonstrated in recent experiments¹⁷ that with moderate frequency (20-Hz) tetanic stimulation of afferent fibre tracts, the most prominent calcium accumulations observed with fura-2 charge-coupled device imaging (1–2 s exposure times) occur in the proximal apical and basal dendrites (Fig. 1b). These AP5-insensitive accumulations are not specific to the dendritic areas that intersect the activated afferents and are probably produced by non-NMDA voltage-dependent calcium channels on the proximal apical and basal dendrites activated by stimulus-induced postsynaptic depolarization. Small AP5-sensitive components of accumulation were observed in postsynaptic dendrites near activated afferent fibres in some experiments, but these accumulations were not statistically reliable for 20 Hz stimulation.

The amplitude and reliability of LTP induction increase with stimulus frequency in the range 0–100 Hz²⁰. Computer simulations suggest superlinear increases of NMDA receptor-mediated postsynaptic calcium accumulations with increasing stimulus frequency²¹. By increasing the temporal resolution of our calcium measurements and increasing the stimulation frequency, we could perform microfluorimetric measurements under conditions more fully optimized for observing NMDA-mediated calcium accumulations. For moderate stimulation (20 Hz for 5 s), our measurement techniques could reliably resolve only an AP5-insensitive component of accumulation in the proximal apical and basal dendrites¹⁷ (Fig. 2b–d). High-frequency stimulation (100 Hz, 1 s) however, resulted in a qualitatively different additional component of accumulation (Fig. 2f) which is greatly reduced in AP5-containing saline (Fig. 2g). The difference map (Fig. 2h) shows that there is a large, localized AP5-sensitive increase in intracellular free calcium in the dendritic zone that intersects the group of activated afferent fibres. This localized calcium accumulation was observed in 19 out of 20 cells with brief (100-Hz) stimulation. Although we could not determine whether each imaged cell experienced LTP, field potential recordings near the imaged cell demonstrated robust LTP of the population spike in all cases (Fig. 2, methods). The average calcium accumulation in distal apical dendrites near stimulated afferents increased from 170 ± 80 nM in AP5-containing saline to 510 ± 230 nM in control saline ($n = 20$). By contrast, basal dendrites showed no AP5-sensitive component: the average accumulation was 300 ± 150 nM in AP5-containing saline and 320 ± 130 nM in control saline. These values are computed from the fluorescent light temporally averaged over the 185 ms required for each image exposure and spatially averaged over a scale of several micrometres: they must be interpreted as an estimated temporal average of the combined calcium changes in both smooth dendrite and dendritic spines.

We next examined whether spatial localization of AP5-sensitive accumulations was dependent on the proximity of activated afferents rather than simply reflecting non-synaptic calcium channels of distal apical dendrites activated by high frequency afferent input. Experiments were conducted with two stimulating electrodes, one activating fibres in stratum radiatum (S1 of Fig.

1a; stimulation of distal apical dendrites), the other activating fibres on the opposite side of the cell body in stratum oriens (S2 of Fig. 1a; stimulation of basal dendrites). Calcium distributions were determined as the cell was synaptically activated with 100 Hz, 0.8 s stimulus trains in either stratum oriens or stratum radiatum, measured in both control saline and AP5-containing saline. For stratum radiatum stimulation (as in Fig. 2h), there was a large AP5-sensitive component of calcium accumulation localized to distal apical dendrites (Fig. 3a, c). However, for stratum oriens stimulation in the same cell there was a large AP5-sensitive accumulation localized to the basal dendrites (Fig. 3b, d). The localization of AP5-sensitive accumulations to the region receiving the activated afferents with smaller AP5-insensitive accumulations located in the proximal apical and basal dendrites was observed in all experiments ($n = 4$) comparing stratum radiatum versus stratum oriens stimulation.

To complement the high spatial resolution CCD images, a low spatial resolution photodiode array and single wavelength fluorescence methods (Fig. 4, methods) were used to provide higher temporal resolution measurements of calcium accumulation. Four photodiodes measured fluorescence from $45 \mu\text{m} \times 45 \mu\text{m}$ areas on basal dendrites, soma, proximal apical dendrites and distal apical dendrites (Fig. 4A). Figure 4B(a–d), shows the time course and AP5-dependence of calcium accumulations at these locations produced by 100 Hz, 1 s activation of an afferent fibre tract that intersected distal apical dendrites. At all dendritic locations a rapid rise in calcium was observed that

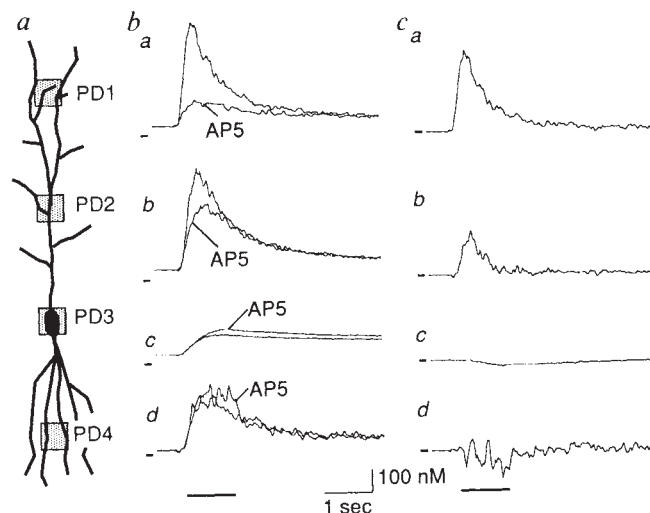


FIG. 4 A, Higher time-resolution measurements of calcium accumulations in four cell areas (PD1–PD4). Individual photodiodes collected fluorescence from a region corresponding to $45 \mu\text{m} \times 45 \mu\text{m}$ areas on the cell image and were separated by $170 \mu\text{m}$. B, The time course of calcium concentration changes produced by 100 Hz, 1 s stimulation of afferent tracts intersecting the distal apical dendrite in control saline and AP5-containing saline in a, distal apical dendrite (PD1); b, proximal apical dendrite (PD2); c, soma (PD3); and d, basal dendrite (PD4). C, AP5-sensitive components of accumulation produced by subtracting the time course of calcium concentration changes in AP5-containing saline from the corresponding time course in control saline for each of the photodiode locations.

METHODS. Photodiode measurements of calcium concentration were made with an array consisting of four photodiodes (Hamamatsu S1087-01) operating in photoconduction mode. Photocurrents were individually amplified (response time: 25 ms) and simultaneously sampled by a microcomputer. Shortly before stimulation, resting calcium concentrations were determined from standard 340 nm, 380 nm ratio measurements of fluorescence at each photodiode. Continuous measurement of fluorescence intensity from constant 380 nm illumination commenced shortly before tetanic stimulation of the Schaffer collateral pathway. Calcium concentrations were computed from the ratio-determined pre-stimulus calcium concentration and the changes in 380 nm fluorescence during the stimulus train by the single wavelength method (see ref. 18; equation 6).

peaked within 200 ms of the onset of the stimulus train, decreasing substantially during the remaining stimulus period. (A commensurate decrease in the population spike amplitude measured during 100-Hz stimulation occurs during this decreasing calcium phase, suggesting reduced postsynaptic cell spiking and depolarization). Accumulations in the soma were somewhat slower and smaller. In AP5 there is a dramatic decrease in the amplitude of the accumulation in distal apical dendrites, the location of afferent stimulation, with little change observed in the accumulation in the basal dendrites. Subtraction of the accumulation measured in AP5 from that in control saline for each photodiode (Fig. 4C a-d) reveals the time course of the AP5-sensitive component of accumulation. All cells tested ($n = 5$) showed large AP5-sensitive calcium accumulations in dendritic regions receiving afferents activated at high frequency. The average peak calcium accumulation (spatially averaged over the $45\ \mu\text{m} \times 45\ \mu\text{m}$ photodiode collection area) in distal apical dendrites near the stimulated afferent fibres increased from 160 nM in AP5-containing saline to 860 nM in control saline, and in several cases reached levels that saturated the fura-2 signal. By contrast, basal dendrites showed only a small AP5-sensitive component (possibly because of enhanced depolarization of voltage-dependent calcium channels): the spatially averaged peak accumulation was 360 nM in AP5-containing saline and 450 nM in control saline.

On the basis of AP5-sensitivity, calcium accumulations observed during synaptic activation can be divided into two components. AP5-insensitive accumulations widely distributed over the apical and basal dendritic trees are produced in response to moderate and high-frequency stimulus trains. Such accumulations could, in principle, play the part of a second messenger in postsynaptic modifications such as heterosynaptic long-term depression²² or in anti-hebbian reductions in synaptic strength²³⁻²⁵. These occur in proximal dendrites regardless of stimulus electrode position, but it is not known if any component of this accumulation is enhanced or localized to dendritic regions near activated afferents. The AP5-sensitive calcium accumulations are strikingly different. They are localized to synaptically activated dendritic regions, verifying a central prediction of the NMDA receptor-mediated induction theory that a pool of calcium is selectively produced that could act locally, either via enzyme activation^{26,27} or some other mechanism^{28,29}, to potentiate only those synapses activated during a high-frequency tetanus. These accumulations are much larger and more reliably observed as the stimulus frequency increases from 20–100 Hz, in parallel with the frequency-dependent efficacy of LTP induction and consistent with computer simulations of NMDA receptor-mediated calcium accumulations²¹. AP5-sensitive accumulations reach their peak at about 200 ms (20 stimulus pulses) after the beginning of high-frequency activation, consistent with the observation that repeated brief trains of high-frequency stimuli are very effective at LTP induction. □

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Stimulation of protein tyrosine phosphorylation by the B-lymphocyte antigen receptor

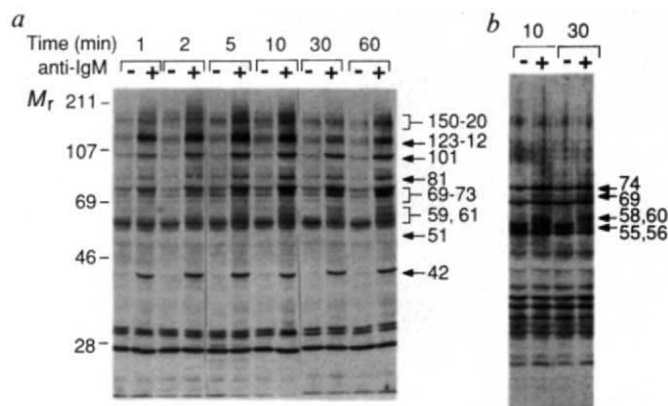
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SIGNALLING by membrane immunoglobulin, the B-lymphocyte antigen receptor, regulates B-cell maturation and activation. Crosslinking of membrane immunoglobulin by antigen or by anti-immunoglobulin antibodies inactivates immature B cells, eliminating many of the B cells capable of producing auto-antibodies¹. By contrast, crosslinking of membrane immunoglobulin promotes activation of mature B cells for clonal expansion and antibody production against foreign antigens². Crosslinking membrane IgM on the immature B-cell line WEHI-231 induces growth arrest³. This response may be analogous to the deletion or inactivation of immature B cells that is induced by antigen or anti-IgM antibodies. Membrane immunoglobulin crosslinking stimulates phosphoinositide hydrolysis, which leads to increases in intracellular calcium and activation of protein kinase C⁴⁻⁶. The induced phosphoinositide breakdown is important for inhibiting WEHI-231 growth (ref. 7 and D. Page, M.R.G., K. Fahey, L. Matsuuchi and A.L.D., manuscript submitted for publication), but may not be sufficient, as agents that elevate calcium and activate protein kinase C cause only partial growth arrest⁷. We now show that in both mature splenic B cells and the immature B-cell line WEHI-231 crosslinking membrane immunoglobulin also stimulates phosphorylation of protein tyrosine, a reaction that has been implicated as a key regulator of cell growth. Most of these phosphorylations were not a consequence of the phosphoinositide pathway. Thus, tyrosine phosphorylation is a second mode of transmembrane signalling by membrane immunoglobulin.

Immunoblot analysis using a monoclonal anti-phosphotyrosine antibody showed that membrane immunoglobulin (mIg) crosslinking induced a rapid increase in protein tyrosine phosphorylation in WEHI-231 cells (Fig. 1a). Within 1–2 min, increased tyrosine phosphorylation of Triton X-100-soluble polypeptides with relative molecular mass of 150,000–200,000 (M_r 150–200K) (four or five bands), 123–129K (a doublet), 101K, 85–90K (two minor bands), 81K, 69–73K, (four bands), and 42K was observed. Maximal phosphorylation occurred within 5–10 min of adding anti-IgM antibodies. Several other polypeptides (two of M_r 59–61K and a minor band at 51K) were tyrosine phosphorylated with slower kinetics. Increased tyrosine phosphorylation was still evident after 1 h of stimulation with anti-IgM antibodies, although for several polypeptides it had declined. Anti-IgM treatment also increased tyrosine phosphorylation of several polypeptides in the Triton X-100-

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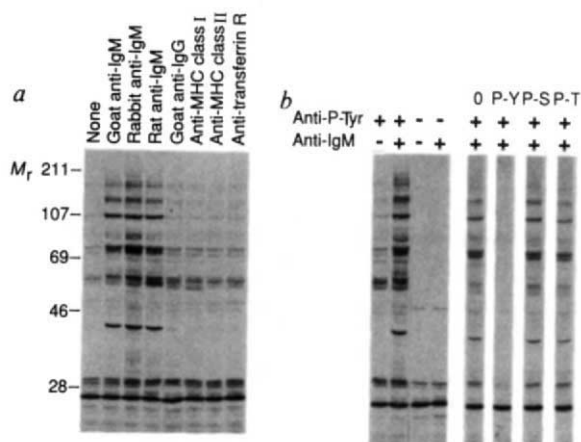
insoluble fraction (M_r 74K, 69K and 58–60K, 2 bands that contains nuclear and cytoskeletal proteins (Fig. 1b). Decreased tyrosine phosphorylation of two 55–56K polypeptides was also observed. These changes in the detergent-insoluble fraction were evident at 5–60 min. Clearly, the increased phosphorylation of polypeptides in the Triton X-100-soluble fraction was not due to translocation of phosphorylated proteins from the detergent-

FIG. 1 Anti-IgM-induced tyrosine phosphorylation in WEHI-231 cells. Anti-phosphotyrosine immunoblot of (a) Triton X-100-soluble fraction and (b) Triton X-100-insoluble fraction of whole-cell lysates from WEHI-231 cells incubated with or without goat anti-IgM antibodies ($5 \mu\text{g ml}^{-1}$, μ -chain-specific, Jackson) for the indicated times. Approximate M_r (K) of the induced bands are indicated to the right of each blot and were estimated using M_r standards that are indicated to the left of each blot.

METHODS. Cells 4×10^6 were resuspended in growth medium⁷ (2 ml) supplemented with 20 mM hepes buffer and incubated 10 min at 37°C before addition of anti-IgM. Reactions were stopped with ice-cold PBS containing 1 mM Na_3VO_4 (8 ml). The cells were pelleted by centrifugation at 4°C , washed twice with PBS-vanadate, and lysed in 0.15 ml lysis buffer (20 mM Tris-HCl, pH 8, 137 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM Na_3VO_4 , 2 mM EDTA, 1 mM PMSF, 20 μM leupeptin, 0.15 U ml^{-1} aprotinin) for 10 min on ice. These conditions have been shown to block *in vitro* phosphorylation and dephosphorylation following cell lysis²⁰. The lysate was centrifuged at $10,000g$ at 4°C for 15 min and the Triton X-100-soluble supernatant was removed. The Triton X-100-insoluble pellet was washed once with lysis buffer, resuspended in SDS-PAGE sample buffer containing 5% 2-mercaptoethanol (0.1 ml), boiled 5 min, and passed 10 times through a 25-g needle. The Triton X-100-soluble lysate was diluted to 1 mg ml^{-1} in lysis buffer containing 62.5 mM Tris-Cl, pH 6.8, 2.3% SDS, and 100 mM DTT. Lysates (35 μg of Triton X-100-soluble protein or 40 μl of Triton X-100-insoluble protein) were separated on 10% SDS-PAGE gels and transferred to nitrocellulose for 3 h at 80 V (0.4 mA) as described in ref. 21. Blots were incubated overnight at 4°C with culture supernatant from an anti-phosphotyrosine hybridoma (4G10, ref. 22, a gift of D. Morrison, B. Druker, and T. Roberts, Dana-Farber Cancer Institute) that was diluted 1:2 in TBS (10 mM Tris-Cl, pH 8, 150 mM NaCl) plus 0.1% Tween-20. The blots were blocked and washed as described in ref. 21. Immunoreactive bands were visualized by incubating the blots for 1 h with goat anti-mouse IgG-alkaline phosphatase (Bio-Rad, diluted 1:700 in TBS plus 0.05% Tween-20), washing and placing them in 0.1 M NaHCO_3 , 1 mM MgCl_2 , pH 9.8 containing 0.5 mg ml^{-1} nitro blue tetrazolium and 0.25 mg ml^{-1} 5-bromo-4-chloro-3-indolyl phosphate 10–20 min. M_r standards (Bio-Rad) were visualized by Ponceau-S staining.

insoluble fraction to the detergent-soluble fraction. Moreover, these changes in tyrosine phosphorylation were not blocked by a 30-min treatment with $100 \mu\text{g ml}^{-1}$ cycloheximide, which completely blocked protein synthesis (data not shown). Thus, the anti-IgM-stimulated changes in tyrosine phosphorylation represent modifications of pre-existing proteins as opposed to translocation or new synthesis.

FIG. 2 Specificity of anti-IgM induction of tyrosine phosphorylation and of immunoblotting procedure. Anti-phosphotyrosine immunoblots of Triton X-100-soluble fraction of WEHI-231 cell lysate. a, WEHI-231 cells were incubated with the indicated antibodies ($5 \mu\text{g ml}^{-1}$) for 10 min and analysed by anti-phosphotyrosine immunoblot. Goat and rabbit anti-IgM antibodies were obtained from Jackson. Note that goat IgG does not bind to mouse Fc receptors for IgG. The rat anti-IgM was affinity-purified monoclonal antibody Bet 1 and the anti-class I and class II MHC antibodies were the mouse monoclonals 15-5-5S and 14-4-4S, respectively. The additional 55K and 53K bands seen with treatment of cells with Bet 1 and 15-5-5S respectively, are the rat and mouse gamma chains, which are recognized by the developing antibody. No additional band was seen with 14-4-4S because WEHI-231 expresses low levels of class II MHC molecules (data not shown). The anti-transferrin receptor antibody was the rat monoclonal R17208.2 which is an IgM antibody. Monoclonal antibodies were obtained and purified as described previously^{6,10}. Immunoblotting methods were as described in Fig. 1 with the exception that 9.5% SDS-PAGE gels were used. b, When the anti-phosphotyrosine antibody was omitted the developing antibody reacted with several polypeptides, but not the ones that were induced by anti-IgM as in a. The 24K band that is recognized by the developing antibody is the immunoglobulin light chain of the WEHI-231 cells. Binding of the anti-phosphotyrosine antibody was blocked by 50 mM phosphotyrosine (P-Y), but not by 50 mM phosphoserine (P-S) or 50 mM phosphothreonine (P-T). The presence of phosphotyrosine in the bands induced by anti-IgM was confirmed by labelling of the cells with $^{32}\text{PO}_4$, followed by immunoprecipitation with the anti-phosphotyrosine antibody and phosphoamino acid analysis by two-dimensional thin layer chromatography²³ of individual bands that had been separated by SDS-PAGE, transferred to Immobilon filters, and hydrolyzed in 6N HCl (data not shown).



Essentially identical results were obtained with three different anti-IgM antibodies (Fig. 2a). Antibodies against class I major histocompatibility complex (MHC) molecules, class II MHC molecules, or the transferrin receptor, all of which are expressed on WEHI-231 cells, did not induce tyrosine phosphorylation. The concentrations of goat anti-IgM antibodies that induced significant tyrosine phosphorylation in WEHI-231 cells corresponded closely to those that cause inositol phosphate production as well as growth arrest (Fig. 3a and data not shown).

The anti-IgM-induced tyrosine phosphorylation could be a consequence of mIgM-stimulated phosphoinositide hydrolysis or an independent signalling pathway. PKC activation and elevation of cytoplasmic calcium, the two known consequences of the phosphoinositide pathway, did not induce the full spectrum of tyrosine phosphorylations that were stimulated by anti-IgM antibodies (Fig. 3a). Activation of PKC by 12, 13-phorbol dibutyrate stimulated the phosphorylation only of the 42K polypeptide and a minor band of 59K. PKC activation stimulates tyrosine phosphorylation of several proteins in B cells⁸. Elevation of cytoplasmic calcium by the calcium ionophore ionomycin neither stimulated tyrosine phosphorylation, nor altered the responses to 12, 13-phorbol dibutyrate. Phosphoinositide breakdown also generates a number of inositol phosphate compounds, which could have second messenger roles. Triggering phosphoinositide breakdown, independent of mIgM, with the G protein-activating agent aluminum fluoride⁹ caused as much phosphoinositide breakdown at 30–60 min as did anti-IgM antibodies (data not shown), but only stimulated tyrosine phosphorylation of the 42K polypeptide and minor bands at 59 and 85K (Fig. 3b). The inability of aluminum fluoride to induce most of the tyrosine phosphorylations implies that mIg-stimulated tyrosine phosphorylation is not a G protein-mediated event, in contrast to mIg-stimulated phosphoinositide breakdown^{10,11} and that mIg-stimulated phosphoinositide breakdown and tyrosine phosphorylation are independent modes of signal transduction.

We found that mIg crosslinking also stimulates tyrosine phosphorylation in mature splenic B cells from BDF₁ mice (Fig. 4).

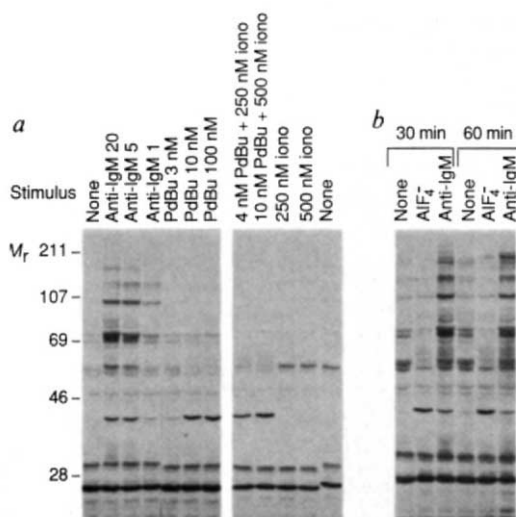


FIG. 3 Anti-IgM-stimulated tyrosine phosphorylation is not a consequence of phosphoinositide hydrolysis. Anti-phosphotyrosine immunoblot analysis of Triton X-100-soluble fraction of WEHI-231 cell lysates. *a*, WEHI-231 cells were incubated with indicated concentrations of goat anti-IgM (in $\mu\text{g ml}^{-1}$) or phorbol 12, 13-dibutyrate (PdBu) and ionomycin (iono) for 10 min. Identical results were obtained after 30 min (data not shown). PdBu and ionomycin did not cause any of the changes in tyrosine phosphorylation in the Triton X-100-insoluble fraction that were induced by anti-IgM (data not shown). *b*, WEHI-231 cells were incubated with $5 \mu\text{g ml}^{-1}$ goat anti-IgM or 30 mM NaF plus $10 \mu\text{M AlCl}_3$ for the indicated times. Aluminum fluoride did not block the anti-IgM-stimulated changes in tyrosine phosphorylation (data not shown). Immunoblotting methods were as described in Fig. 2.

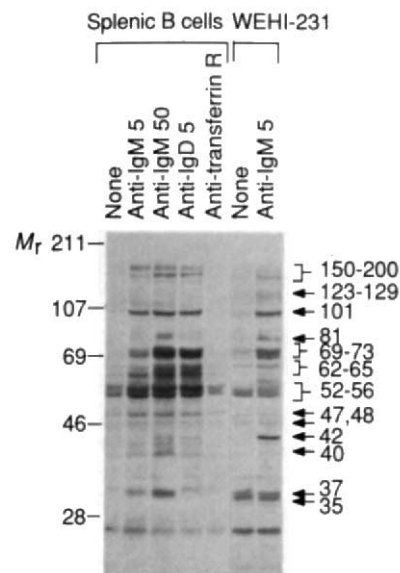


FIG. 4 Anti-IgM and anti-IgD stimulate protein tyrosine phosphorylation in splenic B cells. Anti-phosphotyrosine immunoblot analysis of mature resting B cells incubated 10 min with goat anti- μ -chain-specific antibodies or goat anti-delta chain-specific antibodies (a gift of F. Finkelman, Uniformed Services University for the Health Sciences, Bethesda, Maryland) at the indicated concentrations in $\mu\text{g ml}^{-1}$ or with the rat monoclonal anti-transferrin receptor (R) antibody R17208.2 ($5 \mu\text{g ml}^{-1}$).

METHODS. Resting splenic B cells from BDF₁ mice were prepared by antibody and complement depletion of T cells followed by isolation of small, dense lymphocytes by Percoll density gradient centrifugation²⁴. Flow cytometric analysis showed that this cell population was greater than 95% mlg⁺. The B cells were suspended at $16.7 \times 10^6 \text{ ml}^{-1}$ in a protein-free, modified HEPES-buffered saline buffer described previously⁶, incubated 10 min at 37°C before addition of antibodies for an additional 10 min, then pelleted, lysed and analysed by immunoblot as described in Fig. 1.

Mature B cells express two forms of antigen receptors, mIgM and mIgD, and both anti-IgM and anti-IgD antibodies stimulated the tyrosine phosphorylation of several polypeptides. Many of these had M_r similar to those induced by anti-IgM antibodies in WEHI-231 cells. In addition, anti-IgM and anti-IgD treatment of mature B cells increased tyrosine phosphorylation of polypeptides with M_r of 62–65K, 52–56K and 40–48K, some of which were constitutively phosphorylated in WEHI-231 cells. Interestingly, anti-IgM antibodies, but not anti-IgD antibodies, increased the phosphorylation of a 35K polypeptide although anti-IgD, but not anti-IgM, increased the phosphorylation of a 37K polypeptide. The identities of these differentially phosphorylated proteins are unknown, although p35 could be related to the mIgM-associated protein mb-1 (refs 12, 13).

In summary, crosslinking B-cell antigen receptors induced the rapid tyrosine phosphorylation of several polypeptides in mature B cells and in the immature B-cell line WEHI-231. Most of these phosphorylated polypeptides were present in the cytoplasm or in membranes that were solubilized with Triton X-100. Their appearance was due to induced phosphorylation, rather than relocation within the cell or induced synthesis of constitutively phosphorylated proteins. Given the number of polypeptides and the rapidity with which they are phosphorylated, it is likely that mIg activates a tyrosine kinase as opposed to inhibiting a tyrosine phosphatase or changing the accessibility of the substrates for phosphorylation. Candidates for this kinase include the products of the *src*-like genes *lyn*, *hck* and *blk*, which are expressed in B cells^{14–17}. As most of the mIg-induced tyrosine phosphorylations were not a consequence of the phosphoinositide pathway, the B-cell antigen receptor, like the T-cell antigen receptor^{18,19}, activates two different signalling pathways, phosphoinositide hydrolysis and tyrosine phosphorylation.

Note added in proof. Some of these observations have been made independently by M. Campbell and B. Sefton, (*EMBO J.* (1990) in the press); P. J. L. Lane, F. M. McConnell, G. L. Schieven, E. A. Clark and J. A. Ledbetter, (*J. Immunol.* (1990) in the press) and M. Brunswick, L. E. Samelson and J. J. Mond (manuscript submitted for publication). □

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Cell proliferation inhibited by *MyoD1* independently of myogenic differentiation

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CELL growth and differentiation are usually mutually exclusive¹. Transformation of myoblasts by retroviruses containing the *myc* oncogene inhibits differentiation, preventing cells from withdrawing from the cell cycle^{4,5}. If cell-cycle withdrawal is a prerequisite for myoblast differentiation, it is probably an early event in terminal cell differentiation, but this has not yet been established⁶. *MyoD1* regulates myogenesis⁷. It is expressed only in skeletal muscle⁸, but can convert other cells to muscle cells^{9,10}. The *MyoD1* protein, a nuclear phosphoprotein in part similar to the *myc* family of proteins, is a DNA-binding protein binding to the enhancer sequences of the muscle-specific creatine phosphokinase gene¹¹. Thus, introduction of *MyoD1* into cells provides a simple approach to study the effect of induction of differentiation on cell growth. In cultured NIH 3T3 cells, inhibition of cell proliferation occurs within 18 hours, and expression of myosin starts after 72 hours. Furthermore, injection of *MyoD1* into quiescent NIH 3T3 cells inhibits the serum-induced G0–S phase transition of the cell cycle. In CV1 cells, which are not induced to differentiate by *MyoD1*, DNA synthesis is also inhibited. Expression of *MyoD1* can thus inhibit cell proliferation independently of induction of differentiation. Deletion of the *myc*-like domain in the *MyoD1* gene¹² eliminates the inhibition of DNA synthesis, but substitution of the basic domain with the analogous domain from the E12 transcription factor¹³ inhibits growth yet fails to induce differentiation. Inhibition of DNA synthesis, therefore, seems to be controlled separately from myogenic differentiation.

TABLE 1 Kinetics of inhibition of DNA synthesis and expression of myosin heavy chain in CV1, NIH 3T3, and C3H10T1/2 cells

Time after injection (h)	Cells	N	DNA synthesis inhibition (%)	Myosin (%)
6	CV-1	(108)	–5 ± 2*	
	NIH 3T3	(41)	–8 ± 3	
	C3H10T1/2	(88)	4 ± 1	
12	CV-1	(127)	11 ± 5	(80) 0
	NIH 3T3	(112)	25 ± 2	(79) 0
	C3H10T1/2	(114)	0 ± 4	(111) 0
18	CV-1	(96)	32 ± 1	
	NIH 3T3	(99)	58 ± 8	
	C3H20T1/2	(82)	–4 ± 3	
24	CV-1	(232)	60 ± 4	(170) 0
	NIH 3T3	(261)	75 ± 2	(105) 0
	C3H10T1/2	(214)	38 ± 3	(254) 0
48	CV-1	(98)	77 ± 6	(136) 0
	NIH 3T3	(95)	69 ± 2	(99) 0
	C3H10T1/2	(236)	52 ± 4	(174) 0
72	CV-1	(81)	87 ± 2	(107) 0
	NIH 3T3	(68)	67 ± 1	(65) 6 ± 2
	C3H10T1/2	(47)	54 ± 2	(136) 11 ± 6

Cells plated on coverslips were injected with the pEMC11S plasmid and cultured in DMEM containing 10% FCS for the indicated times. Expression of MyoD1, myosin heavy chain and DNA synthesis were assayed as described in Fig. 1. In the column labelled N, numbers in parentheses indicate the number of MyoD1-positive cells. Myosin percentage indicates the MyoD1-positive cells that contained myosin heavy chain.

* Standard deviation of the mean. The percentage inhibition of DNA synthesis in injected cells was calculated by the formula: % = [% BrdU-positive cells (uninjected) – % BrdU-positive cells (MyoD1 positive)] / [% BrdU-positive cells (uninjected)].

Using an assay system based on microinjection of an expression vector containing the *MyoD1* complementary DNA into cells, we monitored by immunofluorescence: (1) expression of the *MyoD1* protein; (2) expression of the myosin heavy chain; (3) DNA synthesis. *MyoD* was detected with polyclonal-specific antisera, myosin heavy chain with the MF20 monoclonal antibody¹⁴ and DNA with an anti-BrdU (5-bromodeoxyuridine) monoclonal antibody (Fig. 1). *MyoD1* protein was observed in injected cells as a specific nuclear staining as soon as 6 hours after microinjection of the specific plasmid DNA. No *MyoD*-specific staining was observed in uninjected cells or cells injected with the vector alone. When the expression vector containing the *MyoD1* cDNA was injected into growing cells, DNA synthesis was inhibited within 18 hours. Under these growth conditions, the cells did not express myosin until 72 hours after injection. The inhibition of DNA synthesis is thus detected before expression of myosin (Table 1) and the number of arrested cells is larger than the number of myosin-positive cells. The staining pattern with an antidesmin monoclonal antibody (Sigma) could be superimposed on the myosin pattern (not shown). Under growth conditions favouring myogenic

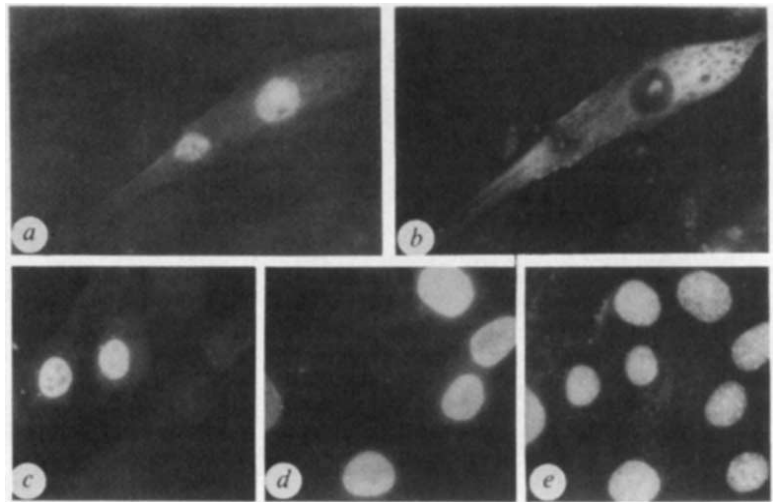
TABLE 2 *MyoD* inhibits serum-induced G0–S phase transition

Time of injection of pEMC11S plasmid relative to serum stimulation (h)	Number of MyoD-positive cells	DNA synthesis inhibition (%)
–24	207	73 ± 6
–6	135	25 ± 1
0	118	15 ± 6
6	140	–8 ± 4

NIH 3T3 cells were incubated for 48 h in Dulbecco's Modified Eagle's Medium (DMEM) containing 0.5% FCS. Cells were then injected with the pEMC11S plasmid 24 hours (–24 h) or 6 hours (–6 h) before, directly after (time 0), or 6 h after (+6 h) serum stimulation. After 18 hours stimulation, cells were given BrdU for 6 hours longer and then fixed and stained for *MyoD1* and assayed for DNA synthesis.

FIG. 1 Expression of MyoD1, myosin and inhibition of DNA synthesis in C3H10T1/2 cells. Cells were seeded on glass coverslips at a density of 10^4 cells per cm^2 and incubated for 48 h in DMEM with 10% FCS and antibiotics. The expression vector pEMC11S (ref. 9), containing the MyoD cDNA under the Moloney sarcoma virus long terminal repeat promoter, was injected into the nuclei of cells with an automated microinjection system (Zeiss) as described previously¹⁸. DNA synthesis in injected cells was monitored by adding BrdU (Sigma, final concentration 100 μM) in the last 6 h of culture, followed by immunostaining. Cells were stained for MyoD (a) and myosin (b) after 72 h; DNA synthesis was scored after 24 h: MyoD (c), BrdU (d) and all nuclei visualized with Hoechst 33258 staining (e).

METHODS. To analyse for DNA synthesis in MyoD1-positive cells, plates were washed once with PBS, fixed for 15 min with 3% paraformaldehyde in PBS, permeabilized with 0.25% Triton X-100, and stained with diluted (1:250) rabbit anti-MyoD1 (ref. 12) for 40 min followed by incubation for 30 min with diluted (1:100) fluorescein-conjugated goat anti-rabbit antibody (Sigma). The cells were incubated for 10 min in 1.5 M hydrochloric acid and stained with monoclonal anti-BrdU (Par-tect, dilution 1:50) followed by a rhodamine-conjugated rabbit anti-mouse antibody (Sigma, dilution 1:100). Cells were finally washed for 5 min in PBS containing Hoechst 33258 (Sigma, final concentration 1 $\mu\text{g ml}^{-1}$) and mounted on glass slides with moviol (pH 7.4). Show here is one field out of an experiment in which 50% inhibition of DNA synthesis was observed in the MyoD-positive cells. To score for myogenesis in MyoD1-positive cells, plates



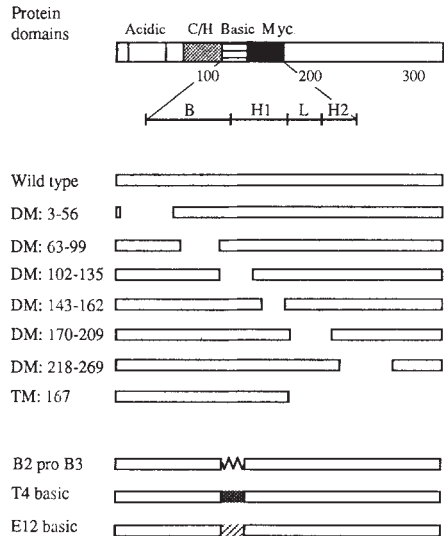
were washed once with PBS, fixed for 5 min with methanol/acetone (1:1) at -20°C and double-stained with anti-MyoD1 (dilution 1:250) and a monoclonal antibody against myosin heavy chain¹⁴ (MF-20, undiluted). The second antibodies were fluorescein-conjugated goat anti-rabbit and a rhodamine-conjugated rabbit anti-mouse, respectively (Sigma, dilution 1:100).

differentiation (2% horse serum instead of 10% fetal calf serum) the number of differentiating C3H10T1/2 and NIH 3T3 cells increases to 37 and 13% of MyoD1-positive cells, respectively, after 72 hours (not shown).

The dissociation between ability to induce withdrawal from the cell cycle and induction of differentiation is supported by experiments with CV1 cells, which do not differentiate in response to expression of MyoD^{9,10}. After microinjection of MyoD into CV1 cells, we observed a large reduction in the number of cells that replicate DNA (Table 1). The percentage of growth inhibition is comparable in all cell lines described in Table 1, but the response to differentiation is variable, being higher in C3H10T1/2, lower in NIH 3T3 and absent in CV1 cells. The slower kinetics of inhibition of cell growth in C3H10T1/2 cells probably results from the longer cycling time of these cells. In separate experiments, we also observed 42% inhibition of DNA synthesis 24 hours after injection of the MyoD1 plasmid into HeLa cells which cannot differentiate through the myogenic pathway¹⁰.

The ability of MyoD protein to inhibit cell proliferation was assayed in a different type of experiment. NIH 3T3 cells were rendered quiescent by culturing them in medium containing 0.5% serum for 48 hours. The MyoD1 expression vector was then injected and the cells stimulated to re-enter the cell cycle by addition of 20% FCS or platelet-derived growth factor (PDGF) at different times before or after microinjection. Table 2 shows that the MyoD1 gene prevents the serum-induced G0-S phase transition. Inhibition of serum-induced activation of growth was observed when the MyoD1 expression plasmid was injected before mitogenic activation of cells. Injection after serum stimulation did not inhibit DNA synthesis and such cells can furthermore go through mitosis, indicating that the MyoD1 expression *per se* is not interfering with transition through the S, G2 and M phases of the cell cycle. This result suggests that the MyoD1 protein has to be expressed in the early part of G1 phase of the cell cycle to prevent cells from entering S phase and replicating DNA. Similar results were obtained with PDGF (not shown).

FIG. 2 MyoD1 expression vector and vectors expressing mutated form of MyoD1 were injected into CV1 cells. After 24 h, cells were double-stained with anti-MyoD1 antibodies and with a monoclonal antibody against BrdU to measure DNA synthesis (see Fig. 1 legend). Myosin/MyoD1, percentage of MyoD1-positive cells containing myosin heavy chain; number in parentheses, number of MyoD1-expressing cells assayed by immunofluorescence; NI, no immunoreactivity with anti-MyoD1. Deletion mutants are described in ref. 12. In the substitution mutants, the replaced regions are indicated in the figure and the mutants are described in ref. 16. Specifically, in the mutant B2 pro B3 the alanine in position 114 was replaced by a proline; in the mutant E12 basic the basic region of MyoD from position 102 to 122 was substituted with the equivalent region of the E12 protein¹³; in the mutant T4 basic the basic region of MyoD was substituted with the basic region of the T4 *achaete-scute* gene product¹⁷.



Myosin/MyoD1 (%)	DNA synthesis inhibition (%)
72	(232) 60±4
34	(104) 57±3
18	(76) 72±10
0	N.I.
0	(91) -8±4
87	(183) 36±5
38	(179) 55±5
46	(133) 39±9
0	NI
0	(90) 5±4
0	(156) 39±5

Several deletion mutants of *MyoD* have been generated and it has been shown that both the helix-loop-helix (HLH) and the basic domains are required for induction of differentiation, whereas other regions of the protein seem to be dispensable for myogenic conversion of C3H10T1/2 cells¹². Plasmids expressing these mutants were injected into CV1 cells and their effects on cell proliferation were analysed. The results indicate that all mutants that could induce differentiation can shut off cell proliferation, whereas the mutant with a deleted HLH domain was unable to inhibit cell proliferation (Fig. 2). The mutant containing the deletion of the basic domain could not be evaluated as it is not immunoreactive to the anti-MyoD1 antiserum. Thus the HLH region at least is required for inhibition of DNA synthesis, as it is for myogenic differentiation.

To assess the role of the basic region we used another set of mutants. MyoD1 binds and forms heterodimers with several other similar proteins^{13,15}, such as the one encoded by the *E12* gene¹³, and this interaction seems to enhance the DNA-binding property of MyoD1 (refs 15, 16). The dimerization seems to be mediated by the HLH domain contained in the Myc-like domain. The basic region also seems to be involved in the transactivating ability of MyoD1. We therefore used substitution mutants in which the basic domain of MyoD had been exchanged with the basic domain of other proteins that have the HLH motif¹⁶. The mutant where the MyoD1 basic domain had been substituted with the *E12* domain can still bind to MyoD-specific DNA-binding sequences as homodimers, but cannot induce differentiation¹⁶ as determined by staining with both antimosin and antidesmin monoclonal antibodies (not shown). This mutant can still significantly inhibit cell proliferation (Fig. 2). The substitution of the MyoD basic region with the *Drosophila achaete-scute*-encoded T4 basic region¹⁷ yields a protein that does not induce differentiation, possibly due to inefficient binding to MyoD target DNA as a homodimer¹⁶. Injection of this mutant does not lead to inhibition of DNA synthesis. Thus it seems possible to dissociate the differentiation function from the DNA-synthesis inhibition function in the basic region of MyoD1. As the mutant containing the *E12* basic region, in contrast to the mutant containing the T4 basic region, can by itself bind to the muscle specific enhancer¹⁶, the growth inhibitory effect may reflect their capacity to bind as homodimers to the target DNA.

Several similar transcription factors like *E12*, MyoD1 and the *achaete-scute* gene product with an HLH domain and a basic region can obviously form heterodimers; many of them together could be required for induction of differentiation and/or inhibition of DNA synthesis. It is, therefore, tempting to suggest that interplay between these factors not only controls induction of terminal differentiation, but also initiates or maintains the growth-arrested stage of the differentiating cells. □

Isolation of 3,4-didehydroretinoic acid, a novel morphogenetic signal in the chick wing bud

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THERE is increasing evidence that retinoic acid is a morphogen involved in vertebrate development^{1,2}. This evidence comes in part from studies of the chick wing bud, in which local application of all-*trans*-retinoic acid results in a duplication of the digit pattern along the anteroposterior axis³⁻⁵. Retinoic acid may be only one of several morphogenetic signalling compounds required for limb pattern formation. To identify novel morphogenetically active compounds, fractionated extracts of whole chick embryos were tested for their ability to induce digit pattern duplications. We describe here the isolation of a new activity present in the limb bud, which we have identified as all-*trans*-3,4-didehydroretinoic acid. The 3,4-didehydroretinoic acid is generated *in situ* from retinol through a 3,4-didehydroretinol intermediate. We show that 3,4-didehydroretinoic acid and retinoic acid are equipotent in evoking digit duplications. These findings suggest that there are at least two endogenous retinoids with morphogenetic properties in the chick limb.

About 2,000 3-4-day-old chick embryos were extracted with ethylacetate and methylacetate and the extract was fractionated on a reversed-phase HPLC column (Fig. 1a). Fractions were examined for the presence of an activity that induces wing pattern duplications when locally applied to chick wing buds. Each fraction was concentrated and absorbed on an ion-exchange bead that was then implanted at the anterior wing bud margin of an embryo (Hamburger-Hamilton stage-20)⁵.

Embryo extracts contained two separable duplication-inducing activities eluting at 14 min and 20 min, respectively (Fig. 1a). The later eluting activity (*a*₂) has the same retention time as authentic all-*trans*-retinoic acid (RA) which has previously been shown to be present in limb buds⁶. The earlier activity (*a*₁) coeluted with A₃₅₀ peak A, which was identified as all-*trans*-3,4-didehydroretinoic acid (ddRA; vitamin A₂ acid, for structure see Fig. 4) by the following criteria. We found that A comigrated with authentic ddRA on a reversed phase HPLC column (Fig. 1b). On methylation, A shifted its retention time to that of authentic 3,4-didehydromethyl retinoate (not shown). When methylated A was analysed by gas chromatography/mass spectrometry (GC/MS), the retention time of the predominant ion species coincided with that of authentic ddRA (Fig. 1c). Together these findings indicate that the compound responsible for duplication activity, eluting at 14 min (Fig. 1a), is ddRA. To provide further evidence that ddRA is a morphogenetically active compound, we have treated chick wing buds with synthetic ddRA. Implanting beads soaked in various concentrations of ddRA induced additional digits in a dose-dependent fashion (Fig. 2a). A comparison with dose-response data of RA shows that ddRA is about equipotent with RA.

We next examined whether the morphogenetic activity of ddRA depends on its conversion to RA. Beads impregnated with [³H]ddRA were implanted into wing buds. After 4 h of incubation *in ovo*, the wing buds were removed, extracted and fractionated by HPLC (Fig. 2b). We observed peaks of polar metabolites (<10 min retention time), a peak representing unmetabolized ddRA (15 min) and a small peak (P*) that coeluted with RA (20.5 min). This peak was subjected to iodine-catalysed isomerization (Fig. 2c), which confirmed its identity with RA. We found that <5% of ddRA is converted to RA (Fig. 2b), yet these two compounds have almost identical dose-response curves (Fig. 2a). These observations indicate that

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ddRA can induce additional digits without significant conversion to RA.

Chromatograms of whole-embryo and limb bud extracts exhibit a main A_{350} peak (B in Fig. 1a, b) that elutes shortly before ddRA. Peak B has the same retention time as authentic all-*trans*-3,4-didehydroretinol (ddROH, vitamin A₂ (ref. 7), see

Fig. 1b). Upon acetylation, B coeluted with authentic 3,4-didehydroretinyl acetate (Fig. 1d). The absorption spectrum of extracted and chromatographically purified B is identical to that of authentic ddROH ($\lambda_{\max}$ = 288 nm and 350 nm, in *n*-hexane, dioxane at a ratio of 95:5, spectra not shown). These results establish that limb buds contain ddROH.

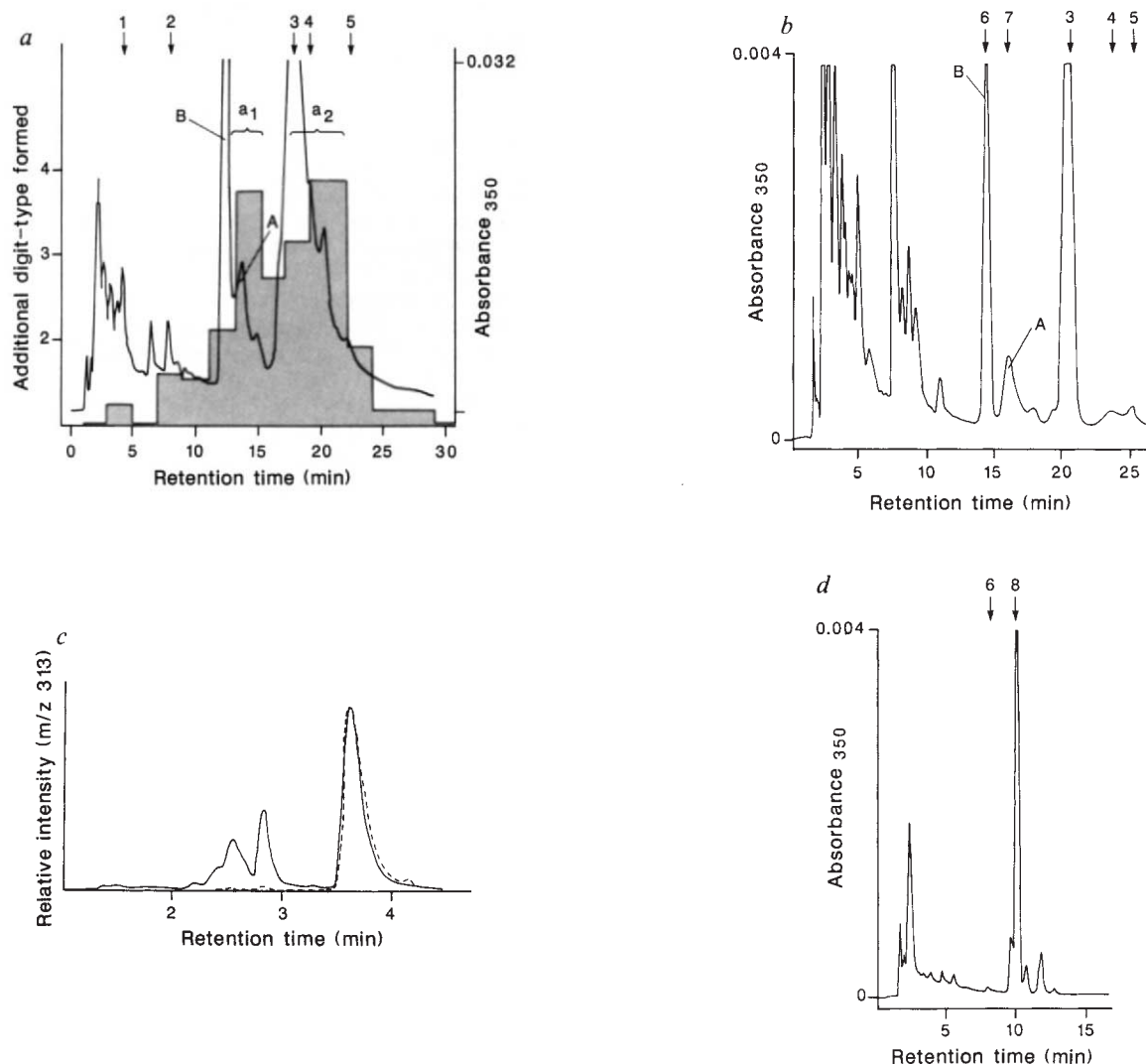


FIG. 1 Detection of a novel morphogenetically active retinoid in chick embryo extracts (a) and sample chromatograms that illustrate its identification as ddRA by HPLC and GC/MS (b, c). a, Absorbance (solid line) and biological activity profile (shaded area) of chick embryo extract fractionated on a C_{18} HPLC column eluted with solvent A. Activity peaks in form of wing patterns with an additional digit 4 were seen in fractions eluting at 14 min (a_1) and 20 min (a_2). b, Fractionation of limb bud extracts of stage-22 embryos (C_{18} column, solvent A). Peaks A and B are base-line separated and coelute with ddRA (arrow 7) and ddROH (arrow 6). c, To further identify peak A, it was methylated and analysed by GC/MS in chemical ionization mode. The chromatograms are from whole-embryo extracts (solid line) or from standard solutions (dashed line) and were generated by monitoring ion current at m/z 313 (MH^+ of authentic 3,4-didehydromethyl retinoate). The minor peak at 2.55 min has a mass spectrum inconsistent with that of a retinoid (not shown), the peak eluting at 2.85 min shows an MH^+ at 313 and may be a *cis* isomer of ddRA. d, Acetylation of peak B results in a peak that coelutes with authentic 3,4-didehydroretinyl acetate (arrow 8, C_{18} column, solvent C). Arrows in Figs 1, 2 and 3 indicate the elution positions of 4-oxo-retinoic acid (1), 5,6-epoxy-retinoic acid (2), ROH (3), RA (4), retinal (5), ddROH (6), ddRA (7), 3,4-didehydroretinyl acetate (8), 13-*cis*-retinoic acid (9).

METHODS. Tissue extraction was performed as described earlier with the following modifications⁶. Precipitation on ice was omitted, instead, the residue of the methylacetate/ethylacetate extract was resuspended in a

mixture of water and solvent A (2:1) and extracted three times with an equal volume of *n*-hexane. The combined extracts were evaporated to dryness and redissolved in methanol for HPLC analysis. Activity assay: Fractions obtained from C_{18} -purified whole embryo extracts were transferred into *n*-hexane (ref. 9), concentrated with a stream of nitrogen gas, and dissolved in 10 μ l DMSO. Ten AG1-X2 beads (Biorad) were soaked for 60 min in the DMSO solution and implanted at the anterior margin of the right wing bud of a Hamburger-Hamilton stage-20 embryo. Digit patterns obtained: fraction 1, 234 (6 cases); fr.2, 2234 (2), 234 (4); fr.3, 234 (10); fr.4, 2234 (5), 234 (2); fr.5, 32234 (2), 2234 (1); 234 (4); fr.6, 432234 (2), 32234 (1), 2234 (4), 234 (2); fr.7, 432234 (7), 2234 (1); fr.8, 432234 (3), 32234 (1), 2234 (2), 234 (1); fr.9, 432234 (4), 32234 (1), 2234 (2); fr.10, 432234 (6), 32234 (1); fr.11, 32234 (2), 2234 (4), 234 (1); fr.12, 2234 (2), 234 (6); fr.13, 234 (7). Fraction 7, containing peak A, was purified on a C_{18} column (solvent C), methylated with diazomethane, and subsequently subjected to GC/MS. The analysis was performed as previously described⁶ with the exception that a DB225 silica column was operated at 240 °C. Acetylation of ddROH was carried out as described for ROH in ref. 9. HPLC: Solvent A was acetonitrile/methanol/2% acetic acid in the ratio 6:2:2 and solvent C was methanol/acetonitrile/water/2% acetic acid in the ratio 80:10:9:1. Flow rates for A and C were 1.3 ml min⁻¹ and 1.2 ml min⁻¹, respectively. Microsorb columns from Rainin were used.

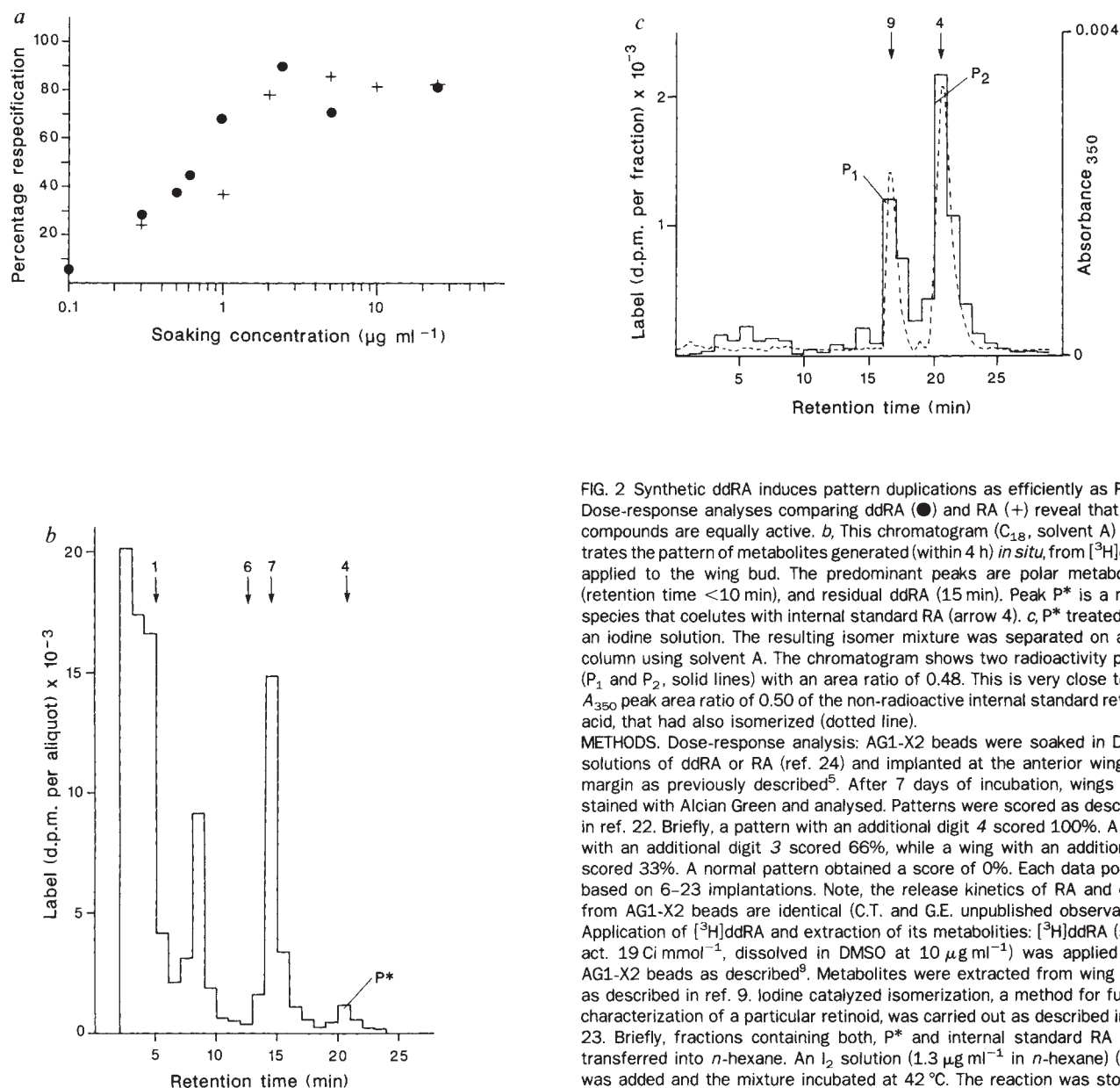


FIG. 2 Synthetic ddRA induces pattern duplications as efficiently as RA. *a*, Dose-response analyses comparing ddRA (●) and RA (+) reveal that both compounds are equally active. *b*, This chromatogram (C_{18} , solvent A) illustrates the pattern of metabolites generated (within 4 h) *in situ*, from $[^3\text{H}]$ ddRA applied to the wing bud. The predominant peaks are polar metabolites (retention time <10 min), and residual ddRA (15 min). Peak P* is a minor species that coelutes with internal standard RA (arrow 4). *c*, P* treated with an iodine solution. The resulting isomer mixture was separated on a C_{18} column using solvent A. The chromatogram shows two radioactivity peaks (P_1 and P_2 , solid lines) with an area ratio of 0.48. This is very close to the A_{350} peak area ratio of 0.50 of the non-radioactive internal standard retinoic acid, that had also isomerized (dotted line).

METHODS. Dose-response analysis: AG1-X2 beads were soaked in DMSO solutions of ddRA or RA (ref. 24) and implanted at the anterior wing bud margin as previously described⁵. After 7 days of incubation, wings were stained with Alcian Green and analysed. Patterns were scored as described in ref. 22. Briefly, a pattern with an additional digit 4 scored 100%. A wing with an additional digit 3 scored 66%, while a wing with an additional 2 scored 33%. A normal pattern obtained a score of 0%. Each data point is based on 6–23 implantations. Note, the release kinetics of RA and ddRA from AG1-X2 beads are identical (C.T. and G.E. unpublished observation). Application of $[^3\text{H}]$ ddRA and extraction of its metabolites: $[^3\text{H}]$ ddRA (spec. act. 19 Ci mmol^{-1} , dissolved in DMSO at $10\text{ }\mu\text{g ml}^{-1}$) was applied with AG1-X2 beads as described⁹. Metabolites were extracted from wing buds as described in ref. 9. Iodine catalyzed isomerization, a method for further characterization of a particular retinoid, was carried out as described in ref. 23. Briefly, fractions containing both, P* and internal standard RA were transferred into *n*-hexane. An I_2 solution ($1.3\text{ }\mu\text{g ml}^{-1}$ in *n*-hexane) (2 ml) was added and the mixture incubated at 42°C . The reaction was stopped after 45 min by adding saturated sodium thiosulfate solution (300 μl). The recovered organic phase was dried down, resuspended in methanol and analysed by HPLC.

We found that the biosynthesis of ddRA involves several steps (see Fig. 4). First, retinol is dehydrogenated at the 3,4 bond of the ionylidene ring to generate ddROH. Dehydrogenation is followed by two sequential oxidations at carbon 15 which convert the hydroxyl group of ddROH, through an aldehyde intermediate, into the carboxyl group of ddRA. To establish that the formation of a double bond at the 3,4 position can occur *in vivo*, we applied $[^3\text{H}]$ ROH locally to wing buds. Extracts prepared from limb bud tissue after 4 h of incubation *in ovo*, displayed two main peaks (Fig. 3a). The first peak coeluted with authentic ddROH, the second with authentic ROH. Limb buds exposed to $[^3\text{H}]$ ddROH generated small amounts of $[^3\text{H}]$ ROH (Fig. 3b). We conclude that limb buds synthesize ddROH from ROH and that under physiological conditions this reaction proceeds predominantly in the forward direction (Fig. 4).

Evidence for oxidation of ddROH to ddRA came from wing buds treated *in ovo* with $[^3\text{H}]$ ddROH. HPLC-fractionated extract contained unmetabolized $[^3\text{H}]$ ddROH (Fig. 3b) and a

radioactive product that coeluted with authentic ddRA on two sequential HPLC columns (Fig. 3b, c). We conclude that ddRA forms locally, by oxidation from ddROH (lower pathway of Fig. 4). Earlier studies have shown that synthesis of RA from ROH follows a similar pathway, possibly involving the same set of enzymatic activities (see upper pathway in Fig. 4)^{8,9}.

We next determined the steady-state levels of retinol, retinoic acid and their 3,4-didehydro analogues during limb development. In the case of ddRA and RA, tissue levels are highest during Hamburger-Hamilton stages 20–22 (Fig. 3d), at which time applied RA can induce additional digits⁴. Moreover, the tissue concentration of ddRA is about six times greater than that of RA, indicating that ddRA is likely to be of physiological significance in limb development (Fig. 3d). The concentration of the precursor molecules ROH and ddROH also vary during development (Fig. 3e) but much less dramatically.

In an earlier study⁵ we noted that induction of a full set of additional digits requires a mean RA concentration in the bud tissue of $\sim 20\text{ nM}$. This is clearly less than the sum of

endogenous RA and ddRA present in buds of stage 21 and 22 embryos (Fig. 3d). However, we wish to emphasize that applied RA attains a concentration of 20 nM only after >14 h of treatment^{5,10}. In contrast, during the first 10 h of treatment (the minimal time required to evoke additional digits¹⁰) the average

level of applied RA is ~90 nM (ref. 10). This is reasonably close to the total concentration of endogenous RA and ddRA in buds at stages 21 and 22 (Fig. 3d).

The scheme in Fig. 4 summarizes the biosynthetic relationship between the known endogenous retinoids in the chick limb bud.

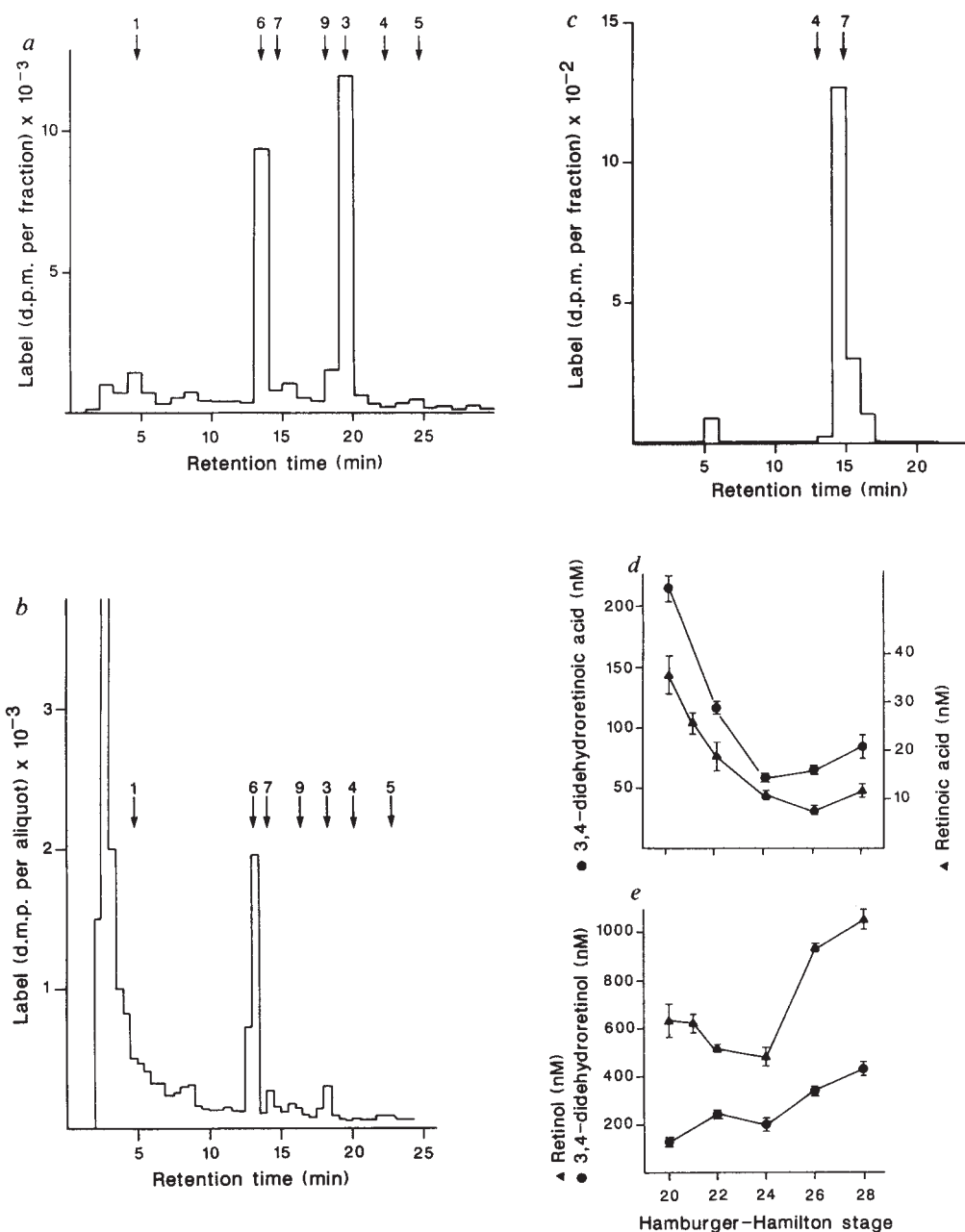
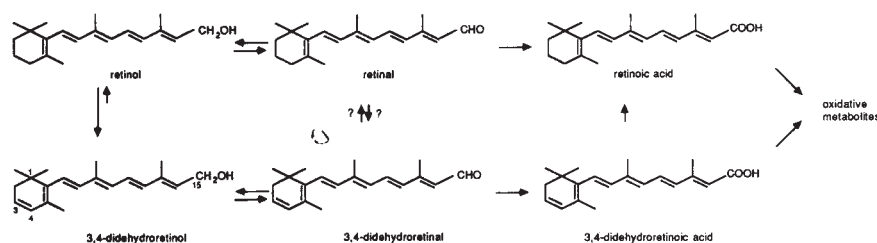


FIG. 3 Interconversion reactions between retinoids (a-c) and changes of endogenous retinoid concentrations during limb development (d, e). a, This sample chromatogram (C_{18} column, solvent A) demonstrates that the principal metabolite of locally applied [3H]ROH is [3H]ddROH (arrow 6). b, Fractionation (C_{18} column, solvent A) of limb bud extract, prepared after 4 h treatment with [3H]ddROH *in ovo*. Comigration of radioactivity with internal standard ddRA (arrow 7) and ROH (arrow 3) indicates that [3H]ddRA and [3H]ROH had formed. Putative [3H]ROH (arrow 3) was subjected to iodine-catalysed isomerization²³. The isomer pattern of putative [3H]ROH and internal standard ROH were identical (not shown). c, Upon rechromatography on a straight phase column (*n*-hexane acetonitrile acetic acid in the ratio 99.5:0.4:0.1, flow rate 2 ml min⁻¹), the fraction containing the putative [3H]ddRA in (b) gave a single radioactivity peak that coeluted with authentic ddRA. d, Both the concentration of RA (▲) and of ddRA (●) are highest during early limb morphogenesis (Hamburger-Hamilton stage 20-22). e, The concentrations of ROH (▲) and ddROH (●) increase as limb morphogenesis progresses. The data points for RA and ROH for stage 21 in (d) and (e) were taken from ref. 6.

METHODS. Endogenous retinoids in limb buds were quantified as described previously⁶. The internal standards used were [3H]ROH (sp. act. 50 Ci mmol⁻¹), [3H]retinal and [3H]RA (both at a sp. act. of 6 Ci mmol⁻¹). Number of measurements performed: stage 20, 4; stage 21, 6; stage 22, 4; stage 24, 2; stage 26, 3; stage 28, 2. To calculate retinoid concentrations, limb bud volumes were determined as described previously²⁴. Local application of [3H]ROH and [3H]ddROH (sp. act. 6 Ci mmol⁻¹) was performed as described in ref. 9. Soaking concentrations were 3.6 mg ml⁻¹ ([3H]ddROH) and 58 µg ml⁻¹ ([3H]ROH). Metabolites were extracted from wing buds as described previously⁹.

FIG. 4 Retinoids in the limb bud form a network. Interconversion between retinoids is indicated by an arrow, the length of which reflects the extent of each reaction as judged from the local-release experiments discussed here and in ref. 9. The explicit evidence for the formation of 3,4-didehydroretinol from 3,4-didehydroretinol and the presence of endogenous retinal and 3,4-didehydroretinol will be reported elsewhere (C.T. and G.E. manuscript in preparation). In previously published chromatograms (see, for example Fig. 5 in ref. 9) we cannot detect conversion of RA to ddRA.



The most notable feature of the network in Fig. 4 are the two parallel synthetic pathways that initiate from the precursor retinol. These pathways lead to two equipotent signalling molecules. The significance of two different forms of retinoic acid in the limb bud is unclear. RA binds to nuclear receptors that function as ligand-regulated transcription factors¹¹⁻¹³. Although a similar finding has not yet been reported for ddRA, it is likely that its role in cell differentiation is mediated by the same mechanism. X-ray structure analysis reveals that a 3,4 double bond will force the ionylidene ring into an almost planar conformation¹⁴, a structural change that could selectively affect its affinity for one of the several retinoic acid receptors or to the cellular retinoic acid binding protein¹⁵ that are expressed in

the limb rudiment (for example, refs 16, 17 and S. Smith and G.E., manuscript in preparation). In addition, the presence of a 3,4 double bond could influence the half-life of the molecule, as degradation of retinoic acid initiates by hydroxylation at the 4 position¹⁸.

A recent study shows that neural tissue from chick embryos can readily convert ROH into ddROH¹⁹. Thus, the ability to generate 3,4-didehydroretinoids from retinol is also characteristic of avian CNS tissue. In addition, human skin contains significant amounts of ddROH, and it has been proposed that ddROH is synthesized locally in the epidermis from ROH^{20,21}. The 3,4-didehydroretinoids may therefore have a broad role in cellular differentiation and in morphogenesis. □

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Polarizing activity and retinoid synthesis in the floor plate of the neural tube

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IN many developing organisms the establishment of axial polarity and the patterning of cells depend on local signals that derive from restricted regions of the embryo¹⁻². In vertebrate embryos, the origins of tissue polarity have been examined extensively in the developing limb³⁻⁵. The anteroposterior pattern of the chick limb seems to be controlled by a morphogen, possibly retinoic acid, that is enriched in a region of the limb known as the zone of polarizing activity (ZPA)⁶⁻¹¹. Certain tissues other than the ZPA have also shown polarizing activity experimentally in the chick limb¹²⁻¹⁴, raising the possibility that signalling molecules involved in pattern formation in different embryonic tissues are conserved. Here we provide evidence that a similar polarizing activity is also present in a restricted region of the developing central nervous system (CNS). We show that a specialized group of neural cells termed the floor plate¹⁵, but not other regions of the CNS, mimics the ZPA in respecifying the digit pattern in the developing chick limb. In addition, using an *in vitro* biochemical assay, we show that the floor plate can synthesize retinoic acid and 3,4-didehydroretinol, the precursor of a second morphogenetically active retinoid, 3,4-didehydroretinoic acid¹⁶. These results show that the floor plate is a local source of a ZPA-like polarizing signal, possibly a retinoid, which may regulate the pattern of cell differentiation in the developing CNS.

The floor plate is an epithelial cell group located at the ventral midline of the developing CNS (Fig. 1) which is believed to have a role in the formation of the neural tube¹⁷ and in the

guidance of developing axons^{15,18,19}. In higher vertebrates, the development of the floor plate seems to depend on two midline structures with roles in early embryonic induction, Hensen's node and the notochord, a mesodermal derivative of the node²⁰⁻²². Fate-mapping studies have shown that the floor plate derives from cells in or around Hensen's node²³⁻²⁶, raising the possibility that some of the specialized properties of Hensen's node and the notochord may be shared by the floor plate. Recently, Hensen's node and the notochord have been shown to have ZPA-like activity when grafted into the chick limb bud¹³. We therefore examined whether the floor plate might be a source of a similar polarizing signal within the neural tube.

To test for polarizing activity, we grafted the floor plate and other regions of the neural tube from stage 16-17 chick embryos under the apical ectodermal ridge of the chick wing bud of stage 20 hosts (Fig. 1a). Embryos were allowed to develop until day 10 and were then analysed for the presence of additional digits. Polarizing activity was quantified using the method of Honig *et al.*²⁷. Grafts of the ventral midline of neural tube, which included the floor plate and a small amount of adjacent neuroepithelium (Fig. 1b), resulted in the formation of additional digits in every case examined (Table 1; Fig. 2a). The polarizing activity of floor plate grafts was 53%, lower than that of the ZPA (78%) but similar to that of Hensen's node (Table 1). Grafts derived from ventrolateral or dorsal regions of the neural tube (Fig. 1b) had no effect on digit pattern (Table 1; Fig. 2b).

At these stages it is difficult to isolate the floor plate completely free of surrounding neural cells, and so it remained possible that the polarizing activity of ventral midline grafts derives from neural cells adjacent to the floor plate. We therefore grafted into the limb bud neural tissue taken from the ventral midline anterior to the floor plate, which terminates in the midbrain²⁸ (Fig. 1c). Grafts of midline or lateral regions of the anterior neural tube had very low polarizing activity (Table 1). Control immunocytochemical experiments with several antibodies to neural antigens show that tissues obtained from all regions of the neural tube survive and differentiate when implanted in the limb bud (not shown). Thus, differences in polarizing activity do not arise from the preferential survival of the floor plate. Taken together, these results suggest that within the neural tube,

TABLE 1 Polarizing activity of developing chick CNS, assayed by limb grafting experiments

Donor stage	Tissue grafted	No. of grafts	Digit pattern				Activity (%)
			234 (normal)	<u>22234</u> or <u>2234</u>	<u>32234</u> or <u>3234</u> or <u>322234</u>	<u>43234</u> or <u>4334</u>	
16-17	Floor plate	37	0	15	22	0	53
	Ventral	19	19	0	0	0	0
	Dorsal	16	16	0	0	0	0
	Anterior midline	24	19	5	0	0	7
	Anterior lateral	29	27	2	0	0	2
	Notochord	5	1	2	2	0	40
24	Floor plate	14	2	4	7	1	50
	Ventral	13	10	3	0	0	8
4-8	Hensen's node	16	3	1	12	0	52
4	Region a	9	7	2	0	0	7
4	Region b	15	12	2	1	0	9
5-7	Notochord	5	0	4	1	0	40
8/8+	Midline	20	20	0	0	0	0
10/11	Midline	17	12	5	0	0	10
20	ZPA	12	1	2	1	8	78

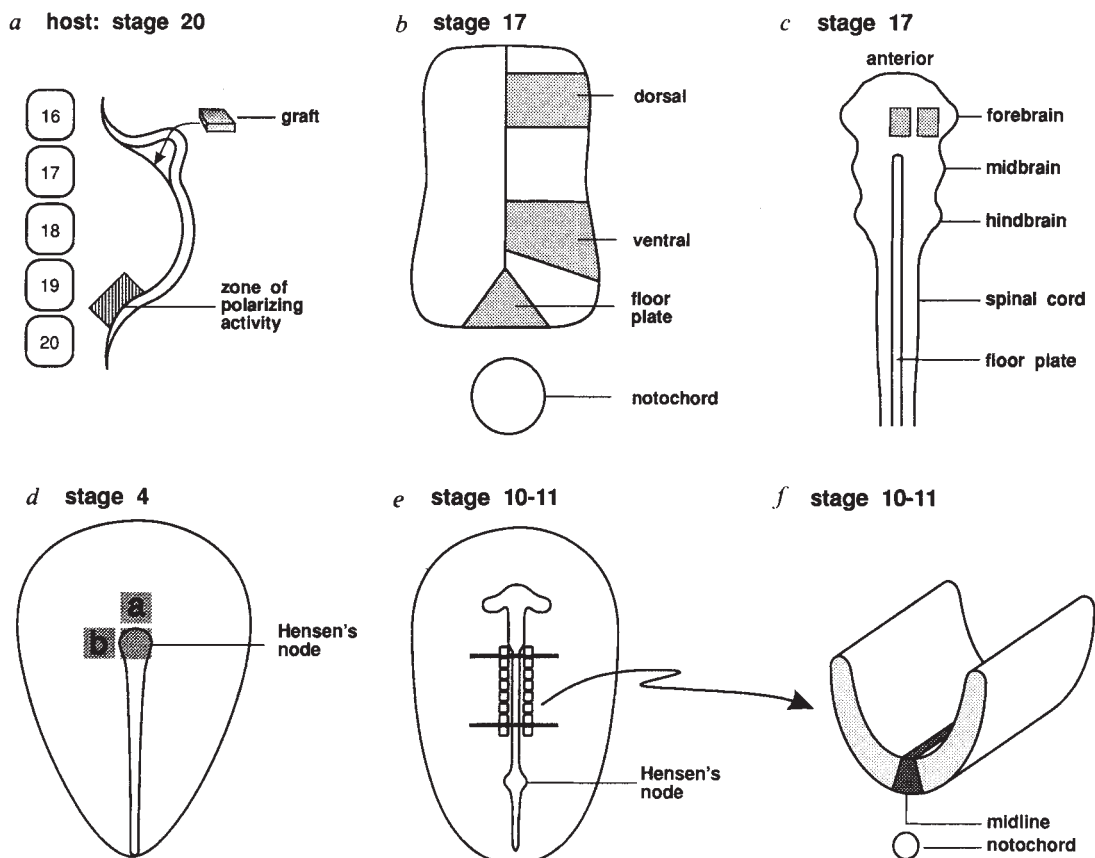
Polarizing activity was determined using the polarizing index described by Honig *et al.*²⁷. Extra digits induced by tissue grafts are shown underlined. Higher per cent activity values indicate greater polarizing activity. After tissue grafts were implanted, host embryos were incubated for a further 7 days at 37 °C. The wings were then removed, fixed in 5% trichloroacetic acid, and stained with Alcian green to make visible the pattern of cartilage elements.

polarizing activity is highly concentrated in or restricted to the floor plate.

We next examined when polarizing activity in the floor plate first appears in relation to the differentiation of the neural tube. Before gastrulation (stage 4), Hensen's node evoked duplica-

tions (Table 1) but prospective floor plate cells in regions of the embryo immediately anterior or lateral to Hensen's node (areas a and b in Fig. 1d) exhibited only very low polarizing activity (Table 1). Polarizing activity was first detected in the midline of the neural plate in stage 10/11 embryos (Table 1) although

FIG. 1 Schematic diagram showing the site of implantation and sources of tissues used in grafting experiments. (Diagrams not drawn to scale.) *a*, Developing wing bud of a stage 20 chick embryo. Grafts were placed under the apical ectodermal ridge of the wing bud, opposite the border between the 16th and 17th somite. The position of the endogenous zone of polarizing activity is marked. *b*, Cross-section of the neural tube of stage 17 chick embryo showing location of tissues used in grafting experiments. Each piece of grafted tissue was approximately 200 µm long. *c*, Diagrammatic view of the anterior part of the CNS of a stage 17 chick embryo. The floor plate terminates within the midbrain of the CNS and is not present at levels anterior to this. Shaded regions show the location of anterior midline and anterior lateral neural tissue used in grafting experiments. *d*, Dorsal view of a Hamburger-Hamilton stage 4 embryo showing the position of Hensen's node and of areas of the epiblast anterior (*a*) and lateral (*b*) to the node that were used in grafting experiments. *e*, *f*, Dorsal view of a stage 10-11 embryo. By this stage Hensen's node has regressed and the neural plate has appeared in midline regions anterior to the node. The neural plate has begun to fold into the neural tube. Floor plate grafts were taken from segments of the neural tube six somites long, dissected and isolated from surrounding



mesoderm. A schematic view of the isolated neural plate from which midline tissue was removed is shown in *f*.

METHODS. Neural tissue from all stages was obtained by dissecting out appropriate areas of neural tube, followed by incubation of the tissue with dispase (1 mg ml⁻¹) for 30 min at 22 °C, to facilitate the removal of surrounding somitic mesoderm and notochord.

the activity was much weaker than at stage 17. Thus, the onset of polarizing activity within the floor plate occurs around the time of neural tube closure but before the onset of neuronal differentiation. Once polarizing activity appears in the floor plate, it persists until at least stage 24, the latest stage examined, whereas other regions of the spinal cord have little or no polarizing activity (Table 1).

Retinoic acid mimics the effects of the ZPA and is a promising candidate for the postulated signalling molecule that patterns the anteroposterior limb axis⁸⁻¹¹. We therefore examined whether the floor plate can synthesize retinoic acid. The small size of the floor plate precluded us from measuring the regional distribution of endogenous retinoids directly. Limb buds, in which the distribution of retinoic acid has been measured¹⁰, are approximately 100 times larger than the floor plate. As an alternative approach, we developed an *in vitro* biochemical assay to examine whether the floor plate can convert retinol into its biologically active metabolite, retinoic acid. Retinoic acid itself is rapidly oxidized to biologically inactive metabolites^{29,30} and thus the absolute amount of retinoic acid generated in these conversion experiments was low (see below). Because of this we also assayed the synthesis of 3,4-didehydroretinol. This retinoid is the predominant metabolite of retinol in limb bud¹⁶ and neural tissue (see below) and is the precursor of 3,4-didehydroretinoic acid, a second morphogenetically active retinoid¹⁶.

The floor plate was dissected from stage 17 neural tube and incubated *in vitro* in chemically defined medium containing 400 nM [³H]retinol for 4 h at 37 °C. This concentration of retinol was chosen because it is close to endogenous levels detected in stage 20 chick limb bud^{10,16}. Radiolabelled retinoids were extracted and identified by sequential HPLC analysis²⁹ (Fig. 3). Separation of extracts on a reverse phase C₁₈ column resulted in two specific peaks of radioactivity (peaks I and II in Fig. 3a). Peak II is primarily unmetabolized retinol, which has a retention time close to that of retinoic acid (Fig. 3a). To determine the amount of retinoic acid synthesized, the fractions coeluting with internal standard retinoic acid were reanalysed on a straight phase column, resulting in a distinct peak of radioactivity which coeluted with authentic retinoic acid (Fig. 3b). Reanalysis of peak I on a straight phase column resulted in a major radioactive peak which coeluted with 3,4-didehydroretinol (Fig. 3c). Retinoic acid and 3,4-didehydroretinol were detected in each of seven separate conversion experiments. The floor plate can also convert retinol to 3,4-didehydroretinoic acid (C.T. unpublished observation). These results show that the floor plate can synthesize retinoic acid and other biologically relevant retinoids. In parallel experiments it was found that the remainder of the neural tube after removal of the floor plate also synthesized retinoic acid, 3,4-didehydroretinol and 3,4-didehydroretinoic acid, but the specific converting activity of the floor plate was somewhat greater (Fig. 3d).

These biochemical results establish that the floor plate can synthesize morphogenetically active retinoids. But retinol-converting activity is only moderately enriched in the floor plate (Fig. 3d). There are at least two possible explanations for the difference between the grafting experiments, which show that polarizing activity is restricted to the floor plate, and the biochemical assays, which show that regions of the neural tube other than the floor plate can synthesize retinoic acid. First, although the concentrations of retinol used in our *in vitro* studies are close to physiological levels^{10,16,30}, there may be differences in the uptake, storage and metabolism of retinoids by neural tissues *in vivo* and *in vitro*. Thus, the levels of retinoic acid detected *in vitro* may not reflect quantitatively the capacity of the same tissues to synthesize retinoic acid *in situ*. The second possibility is that the polarizing activity of the floor plate in the limb bud assay is mediated by a non-retinoid morphogen. Within the limb bud, retinoic acid acting through its nuclear receptors may specify digit pattern by controlling the expression of subordinate genes^{31,32}. The floor plate could directly affect digit

pattern independently of retinoids by acting as a local source of the products of these genes. Recent studies, however, have provided additional evidence linking the floor plate and retinoids. The cellular retinol binding protein (CRBP) is expressed at much higher levels in the floor plate than in other regions of the neural tube³³. In addition, most cells in the chick neural tube, including the floor plate, express retinoic acid receptor transcripts (S. Smith and G.E., manuscript in preparation). Thus, any retinoic acid released by the floor plate is likely to activate these receptors and affect the differentiation of neuroepithelial cells. In support of this, the differentiation and patterning of cells within the neural tube is perturbed by exogenously applied retinoids^{34,35}.

Independent of the identity of the morphogen, the restricted localization of ZPA-like activity in the neural tube provides additional evidence that the floor plate has specialized functions within the developing CNS. In addition to ZPA-like activity, the floor plate orients the growth of developing spinal axons by releasing a diffusible chemoattractant factor^{18,19}. The chemoattractant properties of the floor plate factor are not mimicked by the ZPA, the notochord or retinoic acid (M. Placzek, M. Tessier-Lavigne, T. J. and J. Dodd, unpublished), suggesting that the chemotropic and polarizing activities of the floor plate are distinct.

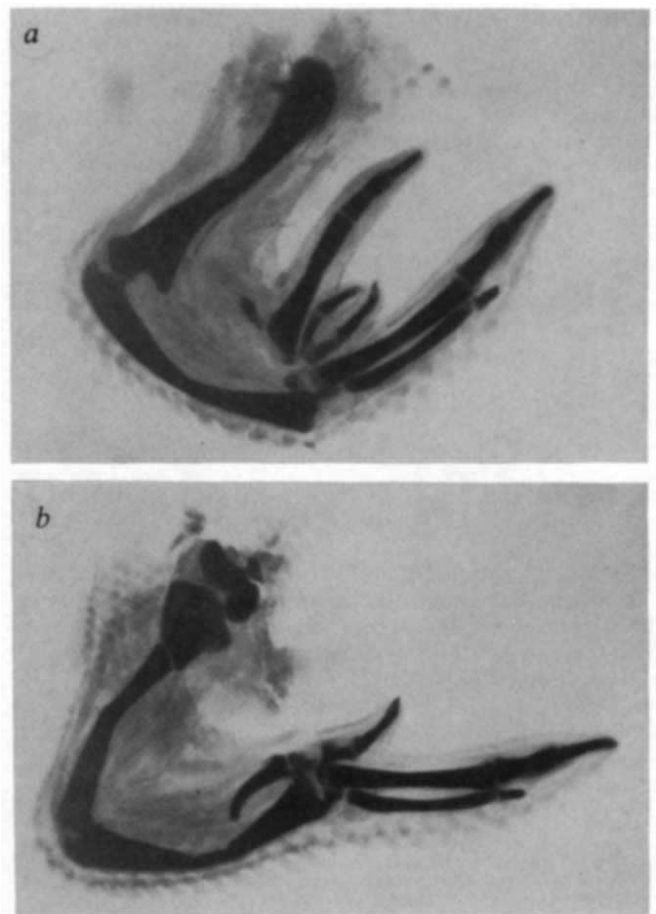
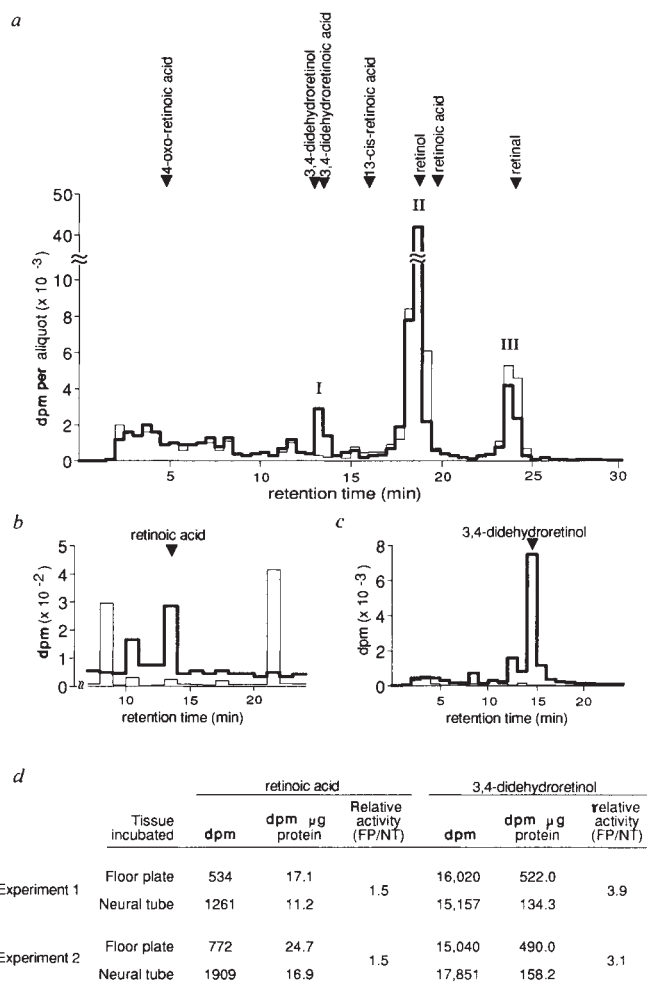


FIG. 2 Dorsal views of whole mounts of right limbs showing the pattern of digits formed by grafting of a small piece of stage 17 floor plate (a) and dorsal neural tube (b) under the ectodermal ridge of stage 20 host chick embryos. Anterior is to the top. The digit pattern in a is 32234 and hence has additional digits 3 and 2 at the anterior margin, whereas the digit pattern in b is normal (234). In both specimens the radius is partially or completely absent. A partial or dysmorphic radius or a loss of the radius was noted with a roughly equal frequency for both types of grafts. For further details see Table 1.

FIG. 3 Sample chromatograms illustrating the metabolic conversion of [3 H]-retinol into [3 H]retinoic acid and [3 H]3,4-didehydroretinol by floor plate tissue from stage 16/17 chick embryos. *a*, Fractionation on a C_{18} HPLC column of metabolites formed after incubation with [3 H]retinol in the presence (thick line) or absence (thin line) of floor plate tissue. Peak I co-elutes with authentic 3,4-didehydroretinol and is formed only in the presence of tissue. Peak II represents unmetabolized retinol. Peak III, which co-elutes with retinal, also appears in the absence of tissue and hence was not further studied. Aliquots measured were 10% of each fraction. *b*, To separate retinoic acid from retinol, fractions from the C_{18} column that eluted between 19 and 21 min were re-analyzed on a straight phase silica column. This results in two radioactivity peaks, the larger of which co-elutes with authentic retinoic acid. The control (thin line) did not show any significant peak at the elution position of retinoic acid. The minor peak of radioactivity is possibly an isomer of retinoic acid. *c*, Rechromatography of peak I on a straight phase silica column results in a radioactivity peak that co-elutes with authentic 3,4-didehydroretinol. Similar chromatographic profiles were obtained in six additional experiments. Retention times of internal standards are indicated by arrows. *d*, Quantitation of conversion of retinol into retinoic acid and 3,4-didehydroretinol by the floor plate (FP) and remainder of the neural tube (NT). Twenty-four pieces of stage 16–17 chick embryo neural tube, each approximately six somites in length, were dissected into floor plate and lateral neural tube before incubation with 400 nM [3 H]-retinol. After 4 h of incubation, retinoic acid and 3,4-didehydroretinol were quantified by HPLC and scintillation counting. The results of two separate conversion experiments are shown. For protein determinations, neural tube and corresponding floor plate tissue was dissected, briefly washed three times, lysed in 50 mM sodium phosphate buffer, pH 7.0, 1% SDS, boiled for 5 min, and the amount of protein determined using the Pierce BCA protein assay reagent kit. The mean protein content of each floor plate piece used in conversion experiments was $1.3 \pm 0.4 \mu\text{g}$ ($\pm$ s.d.; $n=5$ determinations) and of each segment of the remaining neural tube, $4.7 \pm 0.5 \mu\text{g}$ ($\pm$ s.d.; $n=5$ determinations).

METHODS. Tissue pieces (~ 1 mm in length) were dissected from the floor plate and from the remainder of the neural tube of stage 17 chick embryos. After dissection, tissue fragments were preincubated for ~ 1 h in F12 medium lacking serum and supplemented with insulin, transferrin and selenium at 37°C in a CO_2 incubator. Tissue samples were then added in $100 \mu\text{l}$ medium to $11.4 \text{ ng } [^3\text{H}]\text{retinol}$ ($50.1 \text{ Ci mmol}^{-1}$; New England Nuclear) which had been previously dried down in a 1.5 ml microfuge tube under N_2 , to produce a final concentration of $400 \text{ nM } [^3\text{H}]\text{-retinol}$. After incubation for 4 h (37°C ; 5% CO_2), the sample was chilled on dry ice, and a mixture of internal standard retinoids dissolved in ethanol and $200 \mu\text{l}$ stabilizing buffer were added²⁹. The tissue was briefly sonicated and extracted with ethyl acetate/methyl acetate as previously described²⁹. Extracts were first fractionated on a C_{18} column, followed by chromatography on a straight phase silica column (both Rainin Microsorb). The C_{18} column was run at a flow rate of 1.3 ml min^{-1} with acetonitrile/methanol/2% acetic acid in the proportions 60:20:20. The silica column was eluted at 2 ml min^{-1} with either n -hexane/acetonitrile/acetic acid (99.5:0.4:0.1) (*b*) or n -hexane/dioxane (99:5) (*c*).

By analogy with the limb system, the signalling molecules located within the floor plate may regulate the differentiation of adjacent neuroepithelial cells and in this way impose polarity along the dorsoventral axis of the neural tube. More generally, the detection of ZPA-like activity in Hensen's node, the



notochord and the floor plate, three midline structures implicated in inductive signalling in the chick embryo, supports the idea that the strategies³⁶ and signalling molecules used to generate polarity in different embryonic tissues may be conserved. □

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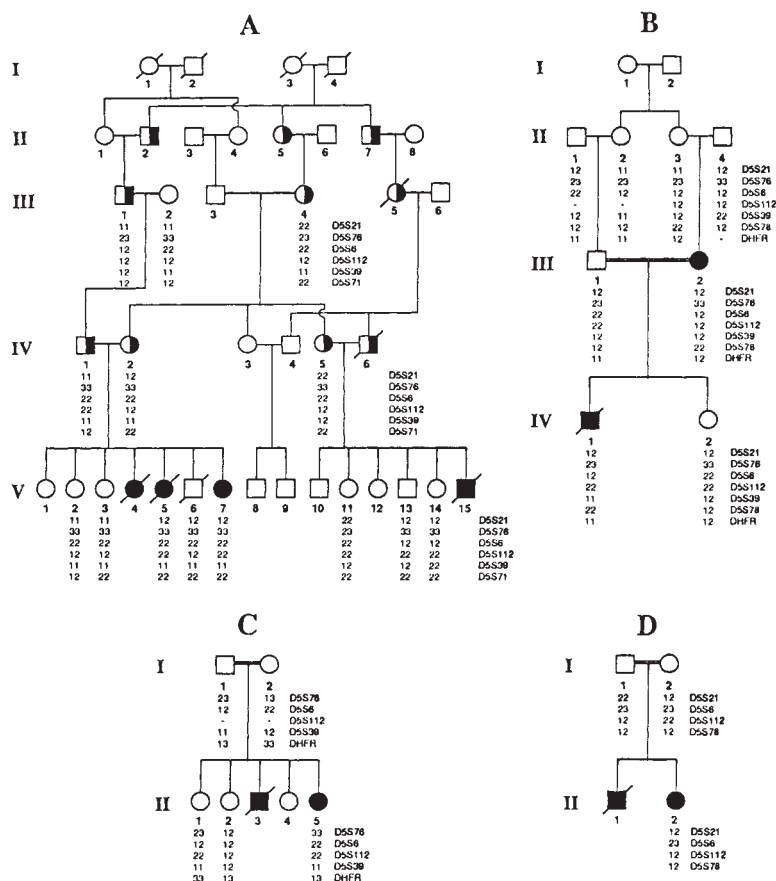
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We calculated multipoint lod scores for the families in Fig. 1 and evaluated the evidence for heterogeneity within the acute and chronic groups by allowing for an admixture of linked and unlinked families in the usual manner (Table 1). Initially, individual III-2 in family B was classified as unaffected to allow for heterogeneity between the two groups. The likelihood ratios in favour of heterogeneity are 3.6 for families with chronic SMA



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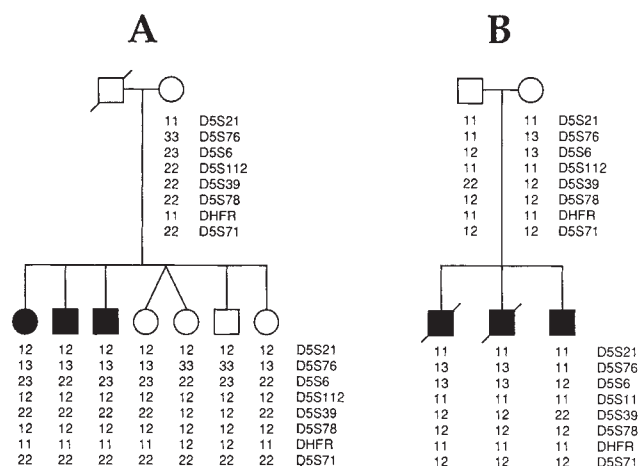


FIG. 2 Family A is diagnosed with chronic SMA and family B with acute SMA. All affected individuals were confirmed by EMG and muscle biopsy analysis. The three affected children in family B are trizygotic triplets. Both families were typed with eight DNA markers and analysed with the other 14 chronic and acute families using the modified HOMOG program¹⁰.

and 2.3 for families with acute SMA, well below the figure of 10 that is usually accepted as meaningful. The likelihood ratio in favour of heterogeneity between the two disease groups is 1.6 (Table 1, calculated in legend). As this is a strong indication of homogeneity between the two groups, we pooled the families with chronic and acute SMA and altered the disease classification of individual III-2 in family B to 'affected'. Within this sample of 16 families, a modification of the HOMOG program¹⁰ yields evidence for heterogeneity, supported by a likelihood ratio of 77.5. One chronic and one acute family (Fig. 2) are identified by the HOMOG program¹⁰ as clearly unlinked, though we caution that only one marker is informative for the single living parent in family A. The maximum lod score (calculated under homogeneity) is 9.26 and occurs at a point 5.8 centimorgans (cM) telomeric of marker D5S39 (see map, Fig. 3). When the two unlinked families are removed from the data set, there is no evidence for heterogeneity and the maximum lod score is 13.16 at a point 0.5 cM telomeric of marker D5S112.

FIG. 3 Multipoint lod scores computed for chromosome 5q markers using the LINKAGE program version 5.03 (ref. 14). In *a*, the dotted line represents analysis of six acute families from our previous study¹. This analysis includes several additional data points and typing with one additional marker, D5S112. The solid line represents analysis of the four consanguineous families in Fig. 1. In *b*, the solid line represents the sum of the two separate analyses assuming genetic homogeneity between acute SMA families. Family C in Fig. 1 is analysed as a consanguineous family only. The bold line is the sum of the two separate analyses, allowing for genetic heterogeneity among acute families, that is, omitting family B (Fig. 2) from the analysis. The arrow marks the corresponding map position of the chronic disease locus inferred from our previous study¹. DNA marker loci are indicated at their corresponding map positions. Recombination fractions between DNA markers were calculated from published map distances¹⁵. For the female-male distance ratio we used the published value of 1.6 as appropriate for this area of the genome¹⁶. Marker JK53 (D5S112) was isolated from a flow-sorted library enriched from chromosome 5 (American Type Culture Collection) and mapped to 5q11.2-13.3 using hybrid cell lines¹⁷. It detects a *PvuII* polymorphism with variable bands of 18 kilobases (kb) (44%) and 4.9 kb (56%) and a constant 3.5 kb band. Typed against CEPH reference pedigrees, it maps 0.5 cM telomeric of marker D5S6.

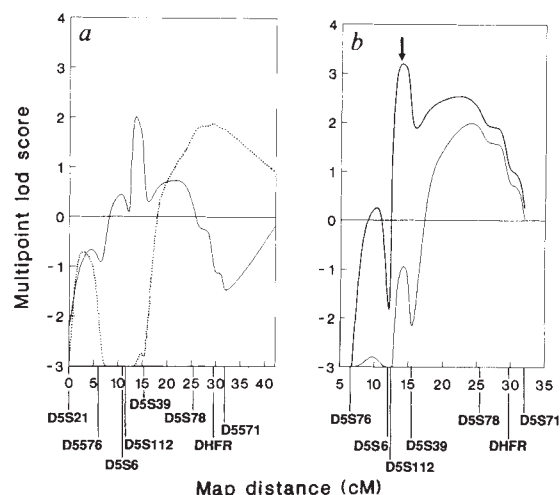
TABLE 1 Assessment of heterogeneity

Groups	Log (L)	α	x
Heterogeneity (H_1)			
Chronic SMA	8.94	0.85	0.124
Acute SMA	2.47	0.70	0.133
Sum	11.41	—	—
Homogeneity (H_0)			
Chronic SMA	—	0.85	0.133
Acute SMA	—	0.70	0.133
Combined	11.21	—	—

Heterogeneity was assessed in the four families from Fig. 1 plus the five acute families and seven chronic families from our previous study¹. To test for heterogeneity between the two groups of families while allowing for possible heterogeneity within each group, a new version of the HOMOG program¹⁰ was used to calculate the log likelihood, $\log(L)$, under the appropriate two hypotheses, H_0 and H_1 , where α is the proportion of families with the disease locus linked to the given markers and x is the estimated map location of the disease locus (marker D5S21 represents map location 0.00). Under heterogeneity between the two groups (H_1), x is allowed to be different in each group and the overall log likelihood is simply the sum shown. Under the homogeneity hypothesis (H_0), the map location of the disease locus is constrained to be at the same point, x , for the two groups; at each x value, $\log(L)$ is maximized over α in each group and added for the two groups; the maximum $\log(L)$ over the points x occurred at the same value as in the families with acute SMA. The antilog of the difference ($11.41 - 11.21 = 0.20$) is 1.6 and represents the likelihood ratio for heterogeneity between the two family groups.

The data from the four consanguineous families is consistent with linkage between acute SMA and chromosome 5q markers. Figure 3a shows the multipoint lod curve for the four consanguineous families (solid line) and the multiply affected families from our previous study¹ (dotted line). Figure 3b shows the summed curve of the combined data set, assuming genetic homogeneity (solid line), or heterogeneity (bold line), among the nine acute families. Addition of the four inbred families shifts the peak position towards the centromere, between markers D5S39 and D5S78, but does not alter the peak height. When the family identified by the HOMOG program as unlinked (Fig. 2b) is omitted from the analysis, a peak lod score of 3.2 between markers D5S112 and D5S39 is obtained, very close to the location of the lod peak for chronic SMA families calculated in our previous study¹ (arrow, Fig. 3b).

Except for family D, all inbred families show some homozygosity of the chromosomal region spanned by markers D5S76-D5S78. The affected child in family D seems heterozygous throughout this region, but is not identified as unlinked by the



HOMOG program. Family B is of interest because the mother (III-2) is diagnosed with chronic SMA and the child (IV-1) with acute SMA. Presumably, a disease allele from generation I is transmitted to both carrier parents in generation III, then inherited in double copy by individual IV-1. Consistent with this model, the affected child is homozygous for markers D5S39 and D5S78. The affected mother presumably has one copy of the disease allele in common with the affected child, and one heterologous copy inherited from her father. Thus, this family is consistent with allelic variation among chronic and acute cases of SMA. Alternatively, the affected mother could have transmitted a sporadic dominant mutation for SMA, though this is unusual in cases of childhood-onset SMA^{11,12}.

The nine acute and seven chronic SMA families in this and our previous study¹ provide strong evidence in favour of genetic homogeneity between the two disorders. The combination of linkage data and genetic homogeneity make it very likely that the acute form of SMA maps to chromosomal region 5q. One chronic family (Fig. 2, A) and one acute family (Fig. 2, B) seem to be unlinked to 5q markers, although family A might conceivably provide data in support of linkage when more informative markers are identified. It must be noted that SMA can be confused with clinically overlapping neurological disorders²⁻⁶, and that the subdivision between acute and chronic SMA is arbitrary, with overlap between the groups¹³. It is not yet possible to distinguish whether, or to what extent, the data in Fig. 2 support genetic heterogeneity as opposed to disease misclassification among the SMA family set. These issues should

be resolved as additional families are typed with markers from chromosome 5q, and disease phenotype is carefully scrutinized relative to genotype. The results caution against early expectations for comprehensive prenatal diagnosis. □

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Putative protein kinase product of the *Drosophila* segment-polarity gene *zeste-white3*

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THE metameric pattern of the *Drosophila* embryo is regulated by a combination of maternal and zygotic genes. The segment-polarity class of genes are required for the correct patterning within each segmental unit. Mutations in any one of these genes results in deletions and duplications of parts of each segment¹. The segment-polarity genes act coordinately by means of local cellular interactions to assign and maintain an identity for each cell in the segment, and to establish segment boundaries²⁻⁵. Here we describe the molecular characterization of a novel segment-polarity gene, *zeste-white3* (*zw3*). Embryos derived from germ lines that are homozygous for *zw3* mutations (*zw3* embryos) have phenotypes similar to embryos that are mutant for the segment-polarity gene *naked* (*nkd*)⁶. These embryos lack most of the ventral denticles, which are differentiated structures derived from the most anterior region of each segment. We have isolated the *zw3* gene and compared the structure of one maternal and one zygotic transcript encoded by the gene. The *zw3* gene is unique among the segment-polarity genes so far characterized, in that it encodes proteins that have homology to serine-threonine protein kinases. This indicates that *zw3* may play a part in a signal transduction pathway involved in the establishment of cell identity within each embryonic segment.

The *zw3* locus is located at chromosomal position 3B1, adjacent to the *period* (*per*) gene^{7,8}. To identify the *zw3* gene at the molecular level we isolated overlapping phage encompassing 70 kilobases (kb) of genomic DNA distal to *per*. The breakpoints

of two deficiencies, *Df(1)64j4* and *Df(1)64f1* (ref. 8), define the proximal and distal limits⁶, respectively, of a 55-kb region of DNA within which the *zw3* gene must lie (Fig. 1a and b). Transcriptional analyses of this region revealed several related transcripts ranging in size from 2.5 to 6.3 kb (Fig. 1d). Although any one transcript may extend over as much as 40 kb of genomic DNA, all transcripts are detected by a single small genomic fragment (illustrated by filled bar in Fig. 1a) and are transcribed in the same direction (data not shown). The inversion *zw3*^{b12}, an allele of *zw3* (ref. 8), identifies this transcription unit as the *zw3* locus, as the inversion breakpoint disrupts the genomic DNA from which the transcripts are derived (Fig. 1b and c). Transcripts are detected at all developmental stages, including messenger RNA of maternal origin at 0-1 h after egg laying, and are developmentally regulated (Fig. 1d). Using a probe that is common to all the *zw3* transcripts we detected a uniform pattern of expression throughout the preblastoderm embryo and in all germ layers of the gastrulating embryo (Fig. 2). Expression is at its highest level during the period from fertilization through germ band elongation, and decreases during germ band retraction.

Using the genomic fragment that is common to all of the *zw3* transcripts we have isolated complementary DNAs from both ovarian⁹ and embryonic 9-12 h¹⁰ cDNA libraries. We have determined the nucleotide sequence of the largest ovarian cDNA (1,257 bp) and the largest embryonic cDNA (3,660 bp). The ovarian cDNA corresponds to the 2.5-kb maternal mRNA, and the embryonic cDNA is assumed to correspond to a transcript that is first detected at 6 h of embryonic development and that persists to the adult stage.

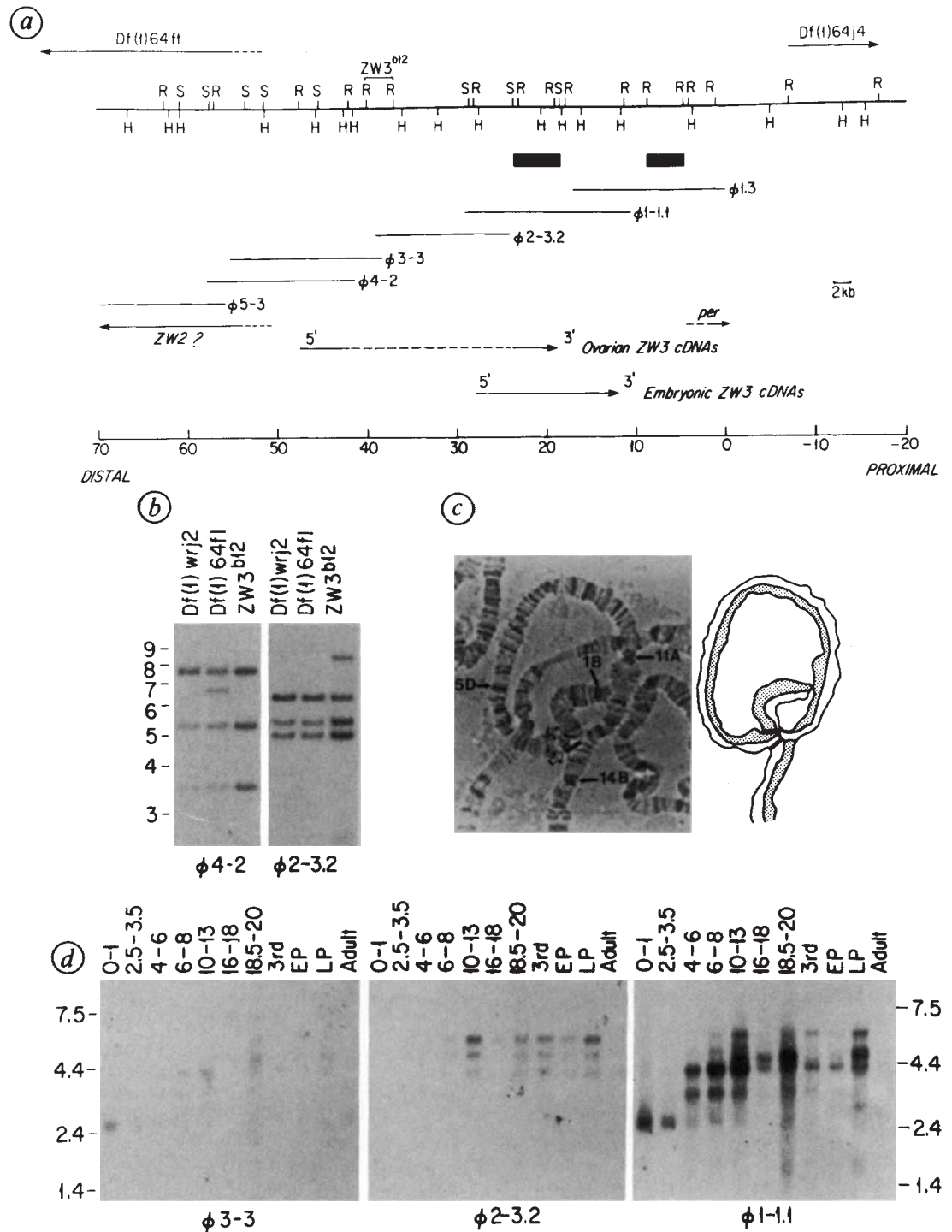
The embryonic cDNA contains a putative open reading frame of 2,199 nucleotides beginning at the ATG at position 378 (Fig. 3b). The context of this initiation codon is not a particularly good match to the *Drosophila* consensus for translation start sites ((C/A)AA(C/A)AUG) (ref. 11); there is another in-frame initiation codon at position 507, which is closer to the consensus. The open reading frame is followed by a 3' untranslated region of 1,057 nucleotides. Several embryonic cDNAs have poly(A)

tracts at their 3' end, preceded by the sequence AAATTA which is a variant of the canonical polyadenylation sequence, AATAAA (ref. 12).

The ovarian cDNA contains an open reading frame of 867 nucleotides beginning with the ATG at position 391 and continuing to the end of the clone (Fig. 3a). This cDNA is not

full-length and lacks sequence from the 3' end of the transcript that it represents. The sequence flanking the initiation codon (TACG) is consistent with the *Drosophila* consensus at the highly conserved second position¹². The ovarian and embryonic cDNAs are identical in a region of protein coding sequence starting at position 476 in the ovarian cDNA and position 1,162

FIG. 1 The molecular organization of the *zw3* locus. **a**, Restriction map and the positions of the deficiencies *Df(1)64j4* (ref. 7) and *Df(1)64f1*, and the inversion *zw3^{b12}* are shown. The lines above the molecular map indicate where the genomic DNA is present in the deficiencies, the stippled portions indicate uncertainty in the position of the breakpoint. The hatched bar represents the probe used to initiate the phage walk and the filled bar represents the probe used to isolate cDNAs. R, *EcoRI*; H, *HindIII*; S, *SalI*. **b**, DNA from heterozygous flies (*Df(1)64f1/FM7* and *zw3^{b12}/FM7*) was digested with *HindIII* and probed with the recombinant phage 4-2 and 2-3.2. Alterations were detected with several restriction enzymes and this data was used to map the breakpoints (data not shown). *Df(1)wrj2* (ref. 8) is a large deficiency that removes DNA in the region of the genome represented by the phage walk. **c**, *In situ* hybridization to polytene chromosomes from 3rd instar larvae of the genotype, *zw3^{b12}/yf*, using the recombinant phage 2-3.2 as a probe. This phage hybridizes to genomic DNA on either side of the inversion, *zw3^{b12}*, which extends from 3B1 to 12F (ref. 8). Open arrows indicate the position of the hybridization and the thin black arrows indicate cytological positions on the chromosome. The stippled region in the panel on the right represents the *yf* chromosome. **d**, Northern-blot analysis of the *zw3* locus. A northern blot containing mRNA from different developmental stages was probed with three overlapping phage that cover the *zw3* locus. The embryonic stages represented are described in hours after egg laying (AEL), the other stages are third instar larvae (3rd), 0–24 h pupae (EP), 96–120 h pupae (LP) and adult.



METHODS. Overlapping phage were isolated from a phage EMBL3 *Drosophila* genomic library²². Sixteen cDNAs were isolated from a LamdaZap (Stratagene) cDNA library made to ovarian RNA⁹ and the longest was sequenced. Embryonic cDNAs were isolated from a λ gt11 library made from mRNA isolated from 9–12 h embryos¹⁰. Eight embryonic cDNAs were isolated and analysed and the longest was chosen for sequencing. Restriction maps have been determined for all of the cDNAs and the ends of all of them have

been sequenced (data not shown). Southern transfer, northern transfer and all hybridizations were performed using standard techniques (Final washes were $0.2 \times \text{SSC}$, $0.1\% \text{ SDS}$ at 65°C)²³. Chromosome squashes for the *in situ* hybridization to polytene chromosomes were as described²⁴, with minor modifications. The recombinant phage 2–3.2 was labelled by nick translation with biotinylated dUTP (ref. 25) and hybridization was detected with the Detek-1-HRP kit (Enzo Diagnostic Inc.).

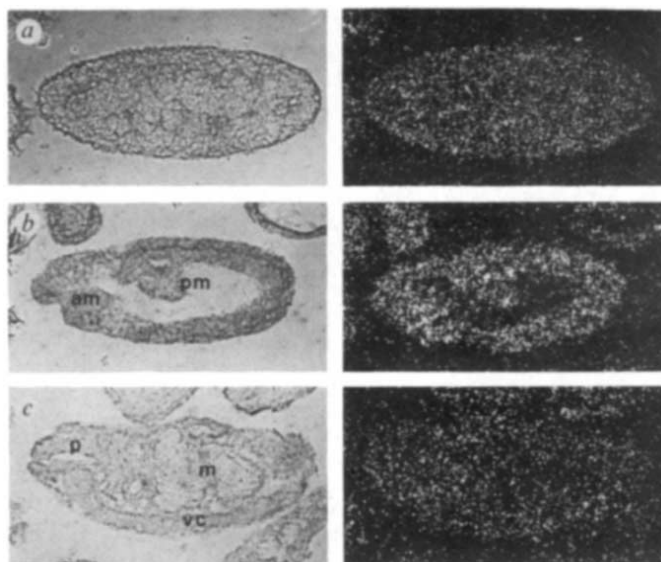


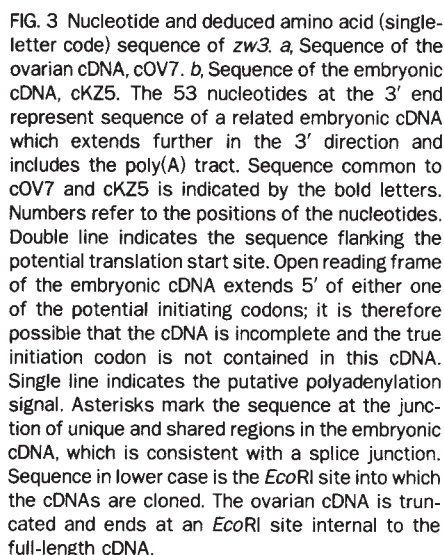
FIG. 2 Localization of *zw3* transcripts. *zw3* transcripts are detected uniformly throughout the embryo at (a) preblastoderm, stage 1–2 (~0–90 minutes AEL), (b) germ-band elongation, stage 11 (~6 hours AEL) and (c) germ-band retraction, stage 15 (~12 hours AEL). Embryos are oriented anterior to the left, dorsal side up. Phase contrast images are on the left, dark-field images on the right. Abbreviations: (am), anterior midgut; (pm) posterior midgut; (p) pharynx; (m) midgut; (vc) ventral cord.

METHODS. Wild-type embryos were collected and grown to the desired developmental stage. Fixation, embedding, sectioning and hybridizations were performed as described²⁶. DNA probes generated from the fragment common to all the cDNAs (Fig. 1) were radioactively labelled by nick translation using [³⁵S]dCTP. Exposure times are 8 days (*a*), 12 days (*b*) and 30 days (*c*).

in the embryonic cDNA, and probably represent alternatively spliced mRNAs. The sequence at position 1,160 of the embryonic cDNA, where the ovarian and embryonic sequence diverge, is consistent with a splice junction¹³ (Fig. 3b).

The sequence that is common to both ovarian and embryonic

cDNAs encodes a protein that resemble the catalytic domain of a serine-threonine protein kinase¹⁴ (Fig. 4). The predicted ovarian protein has 29 amino acids preceding the common domain, and the predicted embryonic protein has a unique amino terminus of 262 amino acids. This putative kinase catalytic



METHODS. The cDNAs were sequenced as double-stranded DNA by the dideoxy chain termination method²⁷ with T7 polymerase (Pharmacia) or Sequenase (USB): cOV7 was sequenced on one strand using nested deletions and oligonucleotides to known sequence; cKZ5 was sequenced on both strands by a combination of subcloning, nested deletions and oligonucleotides.

[illegible]

Draf1	EEILIGPRIGSGSGTGVYRA...HWHGP...VAVKTLNVKTPSPAOLQAF
Cdc28	MSGELANYKRLKLVGGTYGVYKALDLRPGQGQRVVALKKIRLESEDEGVPTA
Cdc2	MENYQKVEKIGEGTYGVYKA...RHKLSGRIVAMKKIRLESEDEGVPTA
zw3	EVSYTDTKIVNGSGFVVFA...KLCDTGELVAIKKVLQDRRFKN....
	=====
Draf1	KNEVAMLK...KTRHCNILLFMGCVSKPS.....LAIVTQWCE...GSSLY
Cdc28	IREISLLKELKDD...NIVRLYDIVHSDAHK...LYLVFEFLDLKRYME
Cdc2	IREISLLKEVNDENNRNRCVRLDLILHAES.K...LYLVFEFLDMLKKYMD
zw3	.RELQIMRKLE...HCNIVKLLYFFYSSEKRDVFLNLVLEYIPETVYKVAR

Draf1	KHVHVSETKFKLNTLIDIGRQVQAQGMDFLHAKNIIHRDLKSNINFLHEDLS.VK
Cdc28	GIPKDP...LGADIVKKFMQCKGIAYCHSHRILHRDLKPNLLINKDGN.LK
Cdc2	RISGTGATSLDPRLVQKFTYVLVNGVNFCHSRRIHRDLKPNLLIDKEGN.LK
zw3	QYAKTKQT.IPINFIRLYMQFLFRSLAYIHSLSGICHRDIKPNLLIDPETAVLK

Draf1	IGDFGLATAKTRWSGEKQANOPTGSILWMAPEVIRMQELNPYSFQSDVYAFGIV
Cdc28	LGDFGLARAAG...VPLRAYTHEIVTLWYRAPEV...LLGGKYSTGVDTSIGICI
Cdc2	LADPGLARSFG...VPLRNYTHEIVTLWYRAPEV...LLGSRHYSTGVDIWSVGC
zw3	LCDPGSAKQLL...HGEFNVSY.ICSRYRAPEL...IFGAINYTTKIDVVSAGCV

Draf1	MYELLAECPLPYGHISNKKDQL.....PMVGRGLLRPDMSQVRSQVRRHSKRLA
Cdc28	FAEMCNRPKIFSGDSEIDQIFKIFRVLGTP.NEAIWPDIVLDPKPSFPQWRR
Cdc2	FAEMIRRSPLFPDSEIDEIFKIFQVLGTP.NEEVWPGVTLLQDYKSTFPWRKR
zw3	LAELLLQGPFIFFGDSGVDQLVEIKVLGTPTREQIREMNPNTYEFK...FPQIKS

Draf1	EDCIKYTP.KDRPLFRPLNML...ENMLRTLPKIHRSASEPNLT
Cdc28	KDLSQVVP.SLDPRGIDLLDKLLAYDPINRISARRAAIHYPQFES
Cdc2	MDLHKVVP.NGEEDATELLSAMLVDPAHRI SAKRALQNYLRDF
zw3	HPWQKVFRIPTTEAINLVSLLETPSARITPLKACAHFPFDEL

domain has the most similarity with the *cdc2* family of genes which includes *cdc2* from *Schizosaccharomyces pombe*¹⁵, its human homologue¹⁶ and *CDC28* from *Saccharomyces cerevisiae*¹⁷. The amino acid comparison of the putative *zw3*-encoded kinase catalytic domain with *cdc2* reveals 34% identity over the entire length of the *cdc2*-encoded protein. The predicted *zw3*-encoded protein contains greater similarity in limited regions of the catalytic domain that seem to be functionally significant; the region of the nucleotide binding site, and two other regions within the catalytic domain that are indicative of a serine-threonine kinase (Fig. 4)^{14,18}. In addition to these specific regions, there are residues that are conserved in the catalytic domain of most protein kinases and that are also conserved in the putative catalytic domain of *zw3* (Fig. 4)¹⁴. Searches of available databases with the unique 5' coding regions of the ovarian and embryonic cDNAs do not reveal significant homology to any other known sequences. We believe that the similarity between *zw3* and *cdc2* reflects the fact that *zw3* encodes a putative serine-threonine kinase rather than its having analogous functions. The two functionally equivalent kinases, *CDC28* from *S. cerevisiae* and *cdc2* from *S. pombe* share >60% identity^{19,20}.

The *zw3* gene encodes several transcripts that are expressed uniformly throughout the embryo. We have sequenced cDNAs representing two developmental transcripts and these encode at least two proteins that resemble serine-threonine kinases. The *zw3* gene may be involved in the signal transduction of information between cells that is required to establish cell identity within each segment²⁻⁵. The nature of the *zw3* gene product and the lack of spatially restricted expression suggests that *zw3* may interact with a gene product that is spatially localized to the anterior portion of each segment, the region affected in *zw3* mutant embryos. The zygotic gene *nkd* is a good candidate for such a gene because mutations in *zw3* and *nkd* result in similar embryonic phenotypes. It is possible that the *nkd* gene product is a substrate, or alternatively an activator, of the putative

FIG. 4 Comparison of *zw3* amino-acid sequence (single-letter code) and other serine-threonine protein kinases. *cdc2*, *S. pombe cdc2*¹⁵; *cdc28*, *S. cerevisiae CDC28*¹⁷; Draf1, the *Drosophila* homologue of the mammalian *c-rat* (refs 28, 29). Comparison of *zw3* and *cdc2* proteins reveals 34% identity over 295 amino acids. Straight vertical line indicates identity between residues. Colon indicates a conservative change, this is based on structural similarity as described¹⁴. Conserved residues in all protein kinases are indicated by asterisks, which mark residues that are conserved in 65 different kinases examined and described¹⁴. Asterisk and a bold letter indicate residues that are identical in 62 of the 65 sequences. Asterisk alone indicates a position where residues of similar structure are conserved in 63 of 65 kinases. Double line indicates the region containing the nucleotide-binding site (GXGXXG) found in many nucleotide-binding proteins, including protein kinases. Single lines indicate sequence that is indicative of a serine-threonine kinase rather than a tyrosine kinase¹⁸. The complete protein sequences for *cdc2* and *cdc28* proteins are shown. Amino acids 265-623 of Draf1 are shown, as are residues 397-688 for the predicted protein of *cKZ5*.

METHODS. Genbank and EMBL databases were searched using the program TFasta of the University of Wisconsin Genetics Computer Group. Alignments were generated using BESTFIT³⁰.

zw3-encoded kinase. This possible interaction between *nkd* and *zw3* remains to be tested, as *nkd* has yet to be cloned and its expression pattern is unknown. In addition to its role in embryonic segmental patterning, *zw3* is required for normal epidermal cell differentiation^{6,21}. In the absence of *zw3* gene product, cells undergo a fate change and assume a neural fate. Zygotic *zw3* gene product may be involved in mediating the choice between neural and epidermal cell fate. The *zw3* gene may be of importance in mediating intercellular interactions that determine cell fate. (The *zw3* locus has recently been renamed *shaggy*²¹. We have used the original nomenclature.) □

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Regulation of the pituitary-specific homeobox gene *GHF1* by cell-autonomous and environmental cues

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HOMEODOMAIN proteins^{1,2} function in determination of mating type in yeast³, segmentation in fruit flies⁴ and cell-type specific gene expression in mammals⁵. In *Drosophila*, expression of homeobox genes is controlled by cell-autonomous interactions between regulatory proteins and environmental cues^{6–8}. Similar controls may operate during mammalian limb development^{9,10} and frog embryogenesis^{11,12}. But, the exact way in which expression of homeodomain proteins is regulated in these systems is not clear and requires biochemical analysis of homeobox gene transcription. We now describe such an analysis of the *GHF1* gene, which encodes a mammalian homeodomain protein specifying expression of the growth hormone (GH) gene in anterior pituitary somatotrophs^{13–15}. *GHF1* is transcribed in a highly restricted manner and the presence of GHF1 protein is correlated both temporally and spatially with activation of the *GH* gene during pituitary development¹⁶. Analysis of the *GHF1* promoter indicates that transcription is also controlled by cell-autonomous interactions involving positive autoregulation by GHF1, and environmental cues that modulate the intracellular level of cyclic AMP and thereby the activity of cAMP response element binding protein (CREB)¹⁷, a ubiquitous transactivator that binds to the *GHF1* promoter.

We examined the regulation of *GHF1* promoter activity by protein factors in cell lines derived from a GH-expressing anterior pituitary tumour (GH3 or GC cells) compared with L cells which do not express GH and can eliminate GH and *GHF1* expression on fusion to GH3 cells¹⁸. *GHF1* was isolated from a rat genomic library¹⁹ and its start site of transcription was

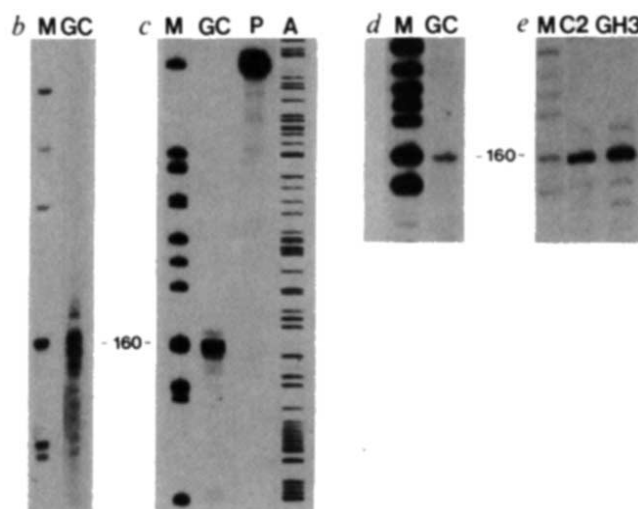
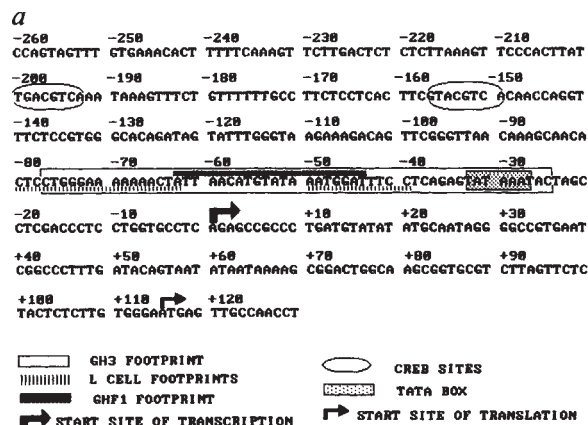


FIG. 1 Identification of the rat *GHF1* promoter. *a*, Nucleotide sequence of the *GHF1* promoter region. The locations of the TATA box, CREB binding sites, a GHF1 binding site and the GH3 and L cell-specific footprints are indicated. *b* and *c*, RNase mapping. Total GC cell RNA was hybridized to 780- or 310-nucleotide (nt) *GHF1* antisense riboprobes complementary to positions -560 to +160 (*b*) or -90 to +160 (*c*), digested with RNase and the protected products were separated on a sequencing gel and visualized by autoradiography. The predominant 160-nt protected fragment corresponds to transcripts initiated 27 bp downstream of the TATA box indicated in *a*. *M*, relative molecular mass (*M_r*) size markers; *P*, undigested probe; *A-A*, specific sequence ladder. *d*, Primer extension analysis of total GC cell RNA with a *GHF1* specific primer. Primer extended products were separated on a sequencing gel and visualized by autoradiography. *e*, Primer extension analysis of *GHF1* RNA transcribed *in vitro*. The p2.0GHF1-BS template was incubated with either C2 or GH3 whole cell extracts to generate *in vitro* synthesized *GHF1* RNA. The same *GHF1* primer used in *d* was used to analyse the RNA synthesized *in vitro*.

METHODS. Plaque-forming units (1×10^6) of a rat genomic library¹⁹ were

screened with a *GHF1* cDNA probe¹³. Duplicate nitrocellulose filters were washed stringently (50 °C, in 0.1 × SSC, 50% formamide) and positive phages were rescreened with a *GHF1* cDNA 5'-specific probe. Of 16 positive clones, three hybridized to the 5' probe and the largest was subsequently subcloned into pBluescript (BS, Stratagene) as *EcoRI-EcoRV*, *EcoRV-EcoRV* and *EcoRV-SalI* fragments. Only the 2.0 kb *EcoRI-EcoRV* subclone (p2.0GHF1-BS) hybridized to northern blots of GC cell RNA (data not shown). The complete nucleotide sequence of this fragment was determined on both strands as previously described³². For RNase protection 20 µg total RNA extracted from GC cells was resuspended in 20 µl hybridization buffer in the presence of 1×10^6 c.p.m. of the different riboprobes³². Probes were synthesized from p2.0GHF1-BS using the T7 RNA polymerase as described by Stratagene. The 780-nt probe was generated by linearizing with *SacI*(-560), whereas the 310-nt probe was generated by linearizing with *HpaI*(-90). Both riboprobes end at the *EcoRI* site at position +160 of *GHF1* followed by 60 nt of Bluescript sequences. Hybridization was for 5 min at 80 °C and overnight at 45 °C. Hybrids were digested as described³² and protected RNA fragments were separated on a 6% polyacrylamide, 7M urea sequencing gel, and visualized by autoradiography. For primer extension analysis, 20 µg total GC cell RNA was hybridized with 20 fmol³²P-labelled *GHF1*-specific primer (5'-GAATTCAGAGGTATAAAGGTATCTGCCGACCTGAAA-GGTTGGAAC-3') in 20 µl of 250 mM KCl, 10 mM Tris buffer pH 7.5, 1 mM EDTA at 55 °C for 1 h. Primer extension was performed as described¹⁸. For *in vitro* analysis whole cell extracts were prepared as described¹⁸. Subclone p2.0GHF1-BS (100 ng) was incubated with 75 µg of C2 or GH3 whole cell extracts for 15 min on ice in 30 µl of 50 mM HEPES buffer, pH 7.9, 100 mM KCl, 12 mM MgCl₂, 1 mM DTT, 1 mM EDTA, 20% glycerol and 10 U of Promega RNasin. Transcription was initiated by addition of 20 µl of 1 mM (each) rNTP and 5% polyvinylalcohol. After 1 h at 30 °C, reactions were stopped by addition of 150 µl transfer RNA stop mix (200 mM NaCl, 20 mM EDTA, 1% SDS, 5 µg tRNA) and analysed by primer extension as described above with the *GHF1*-specific primer.

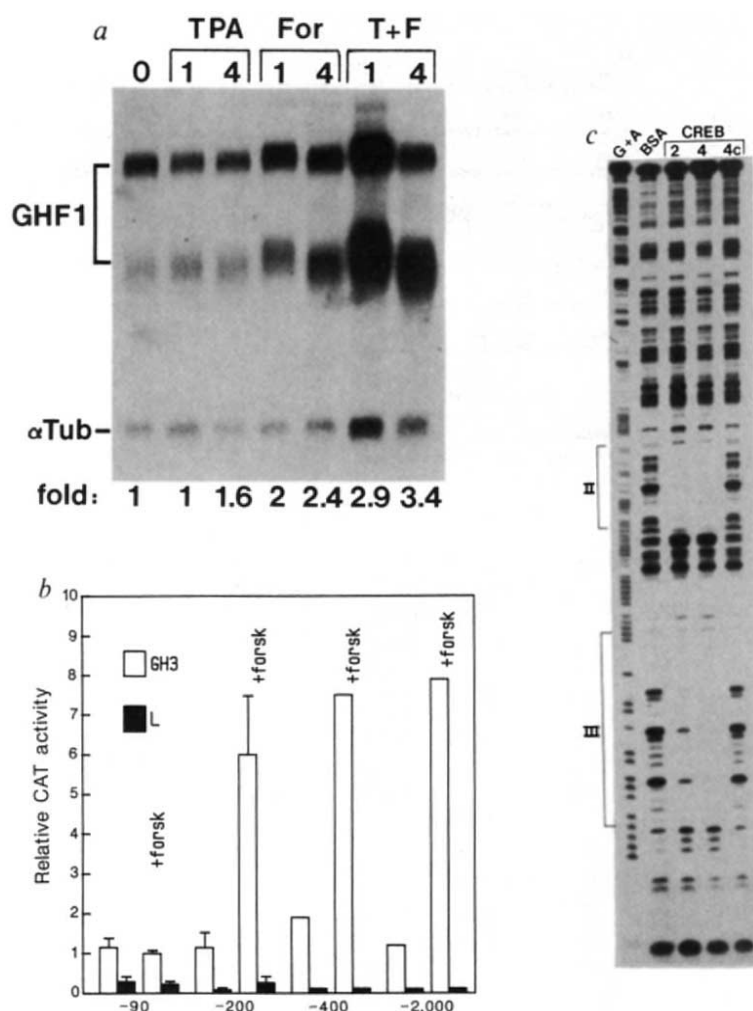


FIG. 2 Cell-type specificity and cAMP inducibility of the *GHF1* promoter. **a**, Northern blot of RNA isolated from GH3 cells. About 1 μ g poly(A)⁺ RNA was loaded in each lane. The *GHF1* probe was a rat cDNA clone in pBluescript transcribed with T3 RNA polymerase. To control for RNA loading, an α -tubulin (α Tub) probe was used. Cells were treated with 100 ng ml⁻¹ TPA, 10 μ M forskolin (For), or a combination of the two (T + F) for the times indicated (in hours). The extent of induction (fold) was determined by densitometric scanning. **b**, Expression of *GHF1*-CAT constructs in GH3 and L cells. Cells were transfected with 10 μ g *GHF1*-CAT plasmids and 2.5 μ g RSV-Luciferase, as described²³. Forskolin was added to 10 μ M 38–40 h after transfection and cells were collected 5 h later. The amounts of extracts used to determine CAT activity were normalized according to the level of luciferase activity. The results shown are from three different experiments for the -90 and -200 constructs and one experiment for the -400 and -2,000 constructs. **c**, CREB binding to the *GHF1* promoter. A *GHF1* probe, labelled at position -240, was incubated with 2 or 4 μ l of affinity-purified CREB and subjected to footprint analysis as described¹⁸. CREB (4 μ l) resulted in complete protection of the *GHF1* promoter at two sites labelled II and III, whereas addition of excess CREB binding sites derived from the somatostatin promoter abolished protection (lane 4C). A Maxam-Gilbert G + A sequencing ladder was used for *M_r* size markers.

identified by northern blot, RNase protection and primer extension analyses (Fig. 1). Sequencing of the promoter region revealed two possible TATA sequences 0.2 kilobases (kb) upstream of the translational start site (Fig. 1a). Only the proximal TATA box functions as such, as indicated by the start sites of *GHF1* RNA synthesized *in vivo* and *in vitro* (Fig. 1, b–e).

The *GHF1* promoter region contains two possible binding sites for CREB, a cAMP-responsive transcription factor¹⁷ (Fig. 1a). These sites may also be recognized by AP-1, a factor that is modulated by the protein kinase C pathway²⁰. Northern blot analysis confirms that *GHF1* is regulated by cAMP and not by the protein kinase C pathway (Fig. 2a). To determine which sequences mediate this response, *GHF1*-CAT constructs containing progressive 5' deletions of the *GHF1* control region were transfected into GH3 cells and tested for cAMP induction. The same mutants were also used to determine sequences responsible for cell-type specific expression. Constructs containing 90–2,000 base pairs (bp) of *GHF1* 5' sequences were expressed in GH3 but not in L cells (Fig. 2b). The response to cAMP was also cell-type specific and required at least 200 bp of *GHF1* 5' sequences. Footprinting of a *GHF1* DNA probe with affinity-purified CREB revealed binding to two sites located around positions -153 and -197 (Fig. 2c).

GHF1-CAT constructs were examined for transcriptional activity *in vitro* on incubation with extracts of GH3 and L cells and two somatic cell hybrids derived from them: C2(GH⁺) and C6 (GH⁻). RNA synthesized *in vitro* was measured by primer extension. Transcripts were efficiently generated by the GH3

and C2 extracts, but only a low level of transcription by the L and C6 extracts was detected (Fig. 3a). Differential use of the *GHF1* promoter may actually be greater than illustrated in Fig. 3a, because twice as much non-expressing extract had to be used to generate a uniform level of α -globin transcripts, which served as an internal control. Of the *GHF1* 5' flanking region, 200 bp was sufficient for cell-type specific transcription *in vitro*.

The differential activity of the *GHF1* promoter was correlated with its recognition by protein factors. Partially purified whole cell extracts of GH3 and L cells²¹ were incubated with a *GHF1* probe labelled on either the coding (Fig. 3b) or noncoding (Fig. 3c) strands, and treated with DNaseI. Three main footprints were generated by the GH3 extract, two of which, II and III, are shared with the L cell extract, and correspond to the CREB binding sites (see Fig. 2c). A large area from position -25 to -77 (region I) was uniquely protected by the GH3 extract. In this region, the L cell extract generated two smaller footprints from -40 to -50 and from -59 to -80.

The sequence of region I revealed that its distal half is homologous (13 out of 15 bp) to the proximal *GHF1*-binding site of the *GH* promoter²². We examined the possibility that *GHF1* expression was positively autoregulated. *GHF1* produced in *Escherichia coli* bound to the distal half of region I (Fig. 4a). This experiment also suggested that the proximal half of region I is protected by at least one other pituitary-specific factor distinct from *GHF1*. Mutagenesis of the *GHF1* site prevented binding of *GHF1* but did not prevent binding of other factors present in the GC extract (Fig. 4a). Without the binding of *GHF1*, however, protection of the downstream site was weaker.

This raises the possibility that binding of the other factor to this site is augmented by an interaction with GHF1.

Co-transfection of $\Delta 5'$ -200GHF1-CAT with a GHF1 expression vector²³ into HeLa cells resulted in transactivation of the GHF1 promoter (Fig. 4b). The mutant lacking a GHF1 binding site ($\Delta 5'$ -200.1GHF1-CAT) was no longer activated by GHF1. The effect of this mutation on the GHF1 promoter was also examined *in vitro*. The $\Delta 5'$ -200.1 mutant was significantly less active than the truncated wild-type promoter, $\Delta 5'$ -200, in the GH3 and C2 extracts (Fig. 4c). Despite this reduction in activity, the mutant promoter still showed cell-type specificity. Incubation of the wild-type GHF1 promoter with an excess of a synthetic high affinity GHF1 binding site derived from the GH promoter reduced its activity in the GH3 extract to the level of the mutant promoter, which itself was not affected (Fig. 4d). These results indicate that GHF1 binding is important for optimal GHF1 promoter activity but the basal cell-type specific activity of the GHF1 promoter is probably due to binding of at least one other pituitary-specific factor distinct from GHF1.

GHF1 transcription is therefore controlled by at least three primary means: a cAMP-activated transcription factor, autoregulation by its own gene product, and transcriptional activation by another pituitary-specific transcription factor that remains to be identified.

By itself the cAMP-activated transcription factor CREB does not activate the GHF1 promoter. Therefore, the response to

CREB requires an interaction with pituitary-specific factors. Current knowledge of GH gene regulation indicates a link between the binding of GH-releasing factor (GRF), which also acts as a somatotroph-specific growth factor²⁴, to cell surface receptors²⁵, increased intracellular cAMP²⁶, and increased GH transcription²⁷. Several attempts to define the cAMP response elements of the GH gene, however, failed to dissociate these elements from those required for somatotroph-specific expression^{28,29}. The finding that GHF1 expression is regulated by cAMP provides an explanation for GH gene induction by GRF. The mechanism for regulating homeobox gene expression by signal transduction systems in response to specific growth factors is not unique to GHF1 and is likely to apply to control of homeobox gene expression in other biological systems.

GHF1 transcription is also positively autoregulated. Positive autoregulation of certain homeobox genes in *Drosophila* is well established^{30,31}, and may be widespread among genes involved in determination of cell fate. GHF1 is involved in terminal differentiation and so autoregulation ensures that once turned

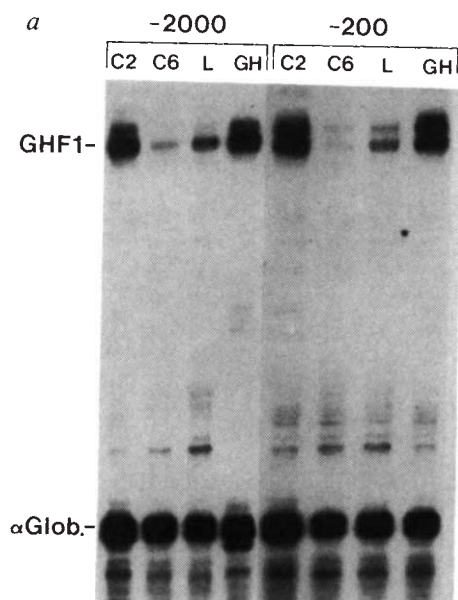
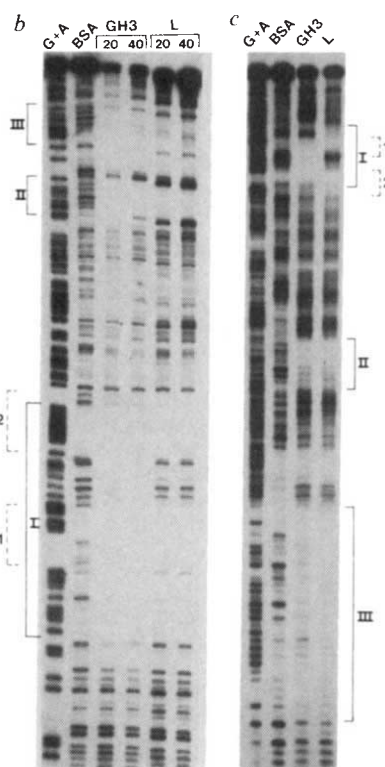
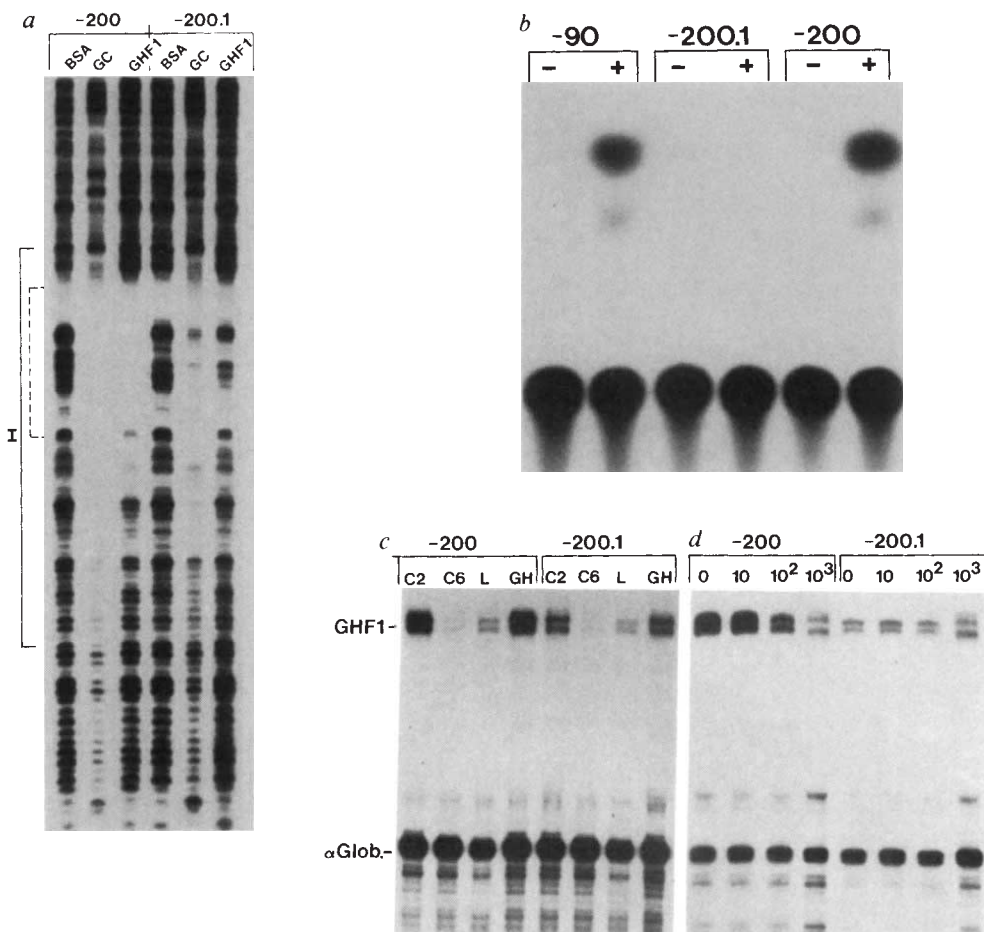


FIG. 3 *In vitro* analysis of the GHF1 promoter. *a*, *In vitro* transcription in whole cell extracts prepared from two GH- and GHF1-expressing (GH3 and C2) and two GH- and GHF1-non-expressing (L and C6) cell lines. GHF1-CAT constructs (100 ng each) containing either 2,000, 200, 90 or 51 bp upstream of the start of transcription were incubated with either 75 μ g (GH3 or C2) or 150 μ g (C6 and L) extracts to generate *in vitro* synthesized RNA. The α -globin (α Glob) gene was included as an internal control to normalize for activity of the extracts and recovery of RNA. Primer extension using CAT and α -globin-specific primers generated 115 nt and 66 nt cDNAs derived from the GHF1 and α -globin transcripts, respectively. Shown are three different experiments in which the transcriptional efficiency of various GHF1-CAT constructs was compared with that of $\Delta 5'$ -200GHF1-CAT. *b*, *c*, Footprint analysis of the GHF1 promoter. GHF1 DNA labelled at position +60 (*b*) or -240 (*c*) was incubated with 20 or 40 μ g of partially purified extracts of GH3 or L cells. A large GH3-specific footprint includes positions -25 to -77 (region I), whereas L cells generate two smaller footprints in this area (footprints 1 and 2). Both extracts protect regions II and III, which include positions -145 to -162 and -187 to -211, respectively.



METHODS. Because of the presence of several AUG codons the 5' untranslated region of GHF1 was deleted before fusing it to the chloramphenicol acetyl transferase (CAT) gene. This was done by the polymerase chain reaction (PCR)³³ using a primer complementary to the GHF1 sequence from -18 to +2 which also contained polylinker sequences to facilitate cloning. The other primers were complementary to GHF1 sequences around positions -200 and -400. After amplification using p2.0GHF1-BS as a template, the amplified fragments were subcloned into pUCAT- which contains CAT coding sequences (M. K., unpublished). A p0.8GHF1-BS construct was generated by deleting at the *SacI* site at position -800. The 2.0 and 0.8 kb fragments were amplified using p2.0GHF1-BS and p0.8GHF1-BS as templates and the downstream GHF1 primer together with an M13 reverse primer. The resulting products were subcloned into pUCAT-. The sequence of the different constructs was determined and found to be the same as the original GHF1 sequence. Footprint analysis was performed as previously described, using whole cell extracts partially purified on a heparin agarose column²¹. The fraction eluting between 0.2 to 0.4 M KCl which contains essentially all of the binding activities²¹ was used.

FIG. 4 Autoregulation by GHF1. *a*, Foot-print analysis. Wild-type (–200) and a mutant (–200.1) *GHF1* promoter probes were labelled at position +60 and incubated with either partially purified GC cell extract or purified GHF1 protein produced in *E. coli*, and analysed by DNaseI footprinting. *b*, Transactivation of the *GHF1* promoter by GHF1. The $\Delta 5'$ -200GHF1-CAT and $\Delta 5'$ -200.1GHF1-CAT constructs (10 μ g each) were co-transfected into HeLa cells with 10 μ g either RSV-neo(–) or RSV-GHF1 (+). Transfections also contained 2.5 μ g RSV-Luciferase as an internal control. After 40 h, cells were collected and luciferase and CAT enzyme activities were determined. Shown are the results of a typical CAT assay using normalized amounts of extracts. *c*, *In vitro* transcription. Activity of a truncated wild-type *GHF1* promoter ($\Delta 5'$ –200) was compared with that of a mutant in the GHF1 binding site ($\Delta 5'$ –200.1) in C2, C6, L and GH3 whole cell extracts. The α -globin template was included as an internal control. *d*, Wild-type (–200) and mutant (–200.1) GHF1-CAT constructs were incubated with 75 μ g of GH3 extract in the presence of 0–10³-fold molar excess of a synthetic GHF1 binding site derived from the *GH* promoter; α -globin was included as internal control. **METHODS.** For expression in *E. coli*, the *GHF1* cDNA was inserted into the pET8C expression vector which uses the T7 RNA polymerase promoter³⁴. After 2-h induction with isopropylthiogalactoside (IPTG), bacterial extracts were prepared and partially purified by precipitation with 20% (NH₄)₂SO₄ and heparin agarose chromatography as described (C. Buchman *et al.*, submitted to *Molec. cell Biol.*). The material was judged to be at least 50% pure by Coomassie blue staining. To generate the –200.1 mutant a *HindIII*–*Sall* fragment containing the wild-type –200 promoter was subcloned



in M13mp19. Single-stranded DNA was prepared and used as a template for mutagenesis as described for the Amersham site-directed mutagenesis system (version 2) with the mutagenic oligonucleotide 5'-GGAAATCCATTTATAAGCTTTAATAGTTTTTC-3'.

on, it will remain active for the duration of the cell's life. GHF1's possible interaction with at least one other pituitary-specific factor to form a more stable complex on the promoter, would lock the *GHF1* gene into an active state. Such tight regulation of genes involved in cell specification could be responsible for irreversible differentiation.

Autoregulation of *GHF1* expression is probably the final step in somatotrophic differentiation. During pituitary development, activation of *GHF1* transcription precedes the appearance of GHF1 protein by several days¹⁶, emphasizing the requirement for at least one other factor, still to be identified, for initial activation. □

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Nucleation and growth of fibres and gel formation in sickle cell haemoglobin

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DEOXYGENATED sickle haemoglobin polymerizes into long 210-Å diameter fibres that distort and decrease the deformability of red blood cells, and cause sickle cell disease. The fibres consist of seven intertwined double strands¹⁻³. They can form birefringent nematic liquid crystals (tactoids)⁴ and spherulites^{5,6}. Rheologically, the system behaves as a gel^{7,8}. The equilibria show a phase separation and a solubility⁹⁻¹⁴. The reaction kinetics show a delay time, are then roughly exponential and are highly dependent on concentration and temperature^{9,10,15-18}, and accord with the double nucleation model^{5,19}. But these conclusions are derived from macroscopic data, without direct observation of individual fibres. We have now used non-invasive video-enhanced differential interference contrast (DIC) and dark-field microscopy to observe nucleation, growth and interaction of sickle deoxyhaemoglobin fibres in real time. The fibres originate both from centres that produce many radially distributed fibres and on the surface of pre-existing fibres, from which they then branch. The resulting network is cross-linked and dynamic in that it is flexible and continues to grow and cross-link. Our results support most aspects of the double nucleation model.

Immediately after a temperature jump, there is a delay during

which no polymers can be seen on scanning a slide of sickle (S) deoxyhaemoglobin. Polymers then appear and grow rapidly. Figure 1*a-d* shows two fibres, with growth rates of 0.116 and 0.113 $\mu\text{m s}^{-1}$ (Fig. 1*e*) corresponding to 250 monomers (haemoglobin tetramers, relative molecular mass 64,500 (M_r , 64K)) per second (based on the 14-stranded structure with a 64-Å repeat¹⁻³). The growth rates in three samples are also shown in Fig. 1*e*. Fibres grew to many times the 40 μm width of the field, many reaching millimetre lengths. Figure 1*f-k* shows growth in dark-field micrographs after thick fibre bundles have been cut with a focused ultraviolet beam²⁰. Both cut ends grow at roughly the same rate.

Our results demonstrate two kinds of nucleation, the first of which is shown in Fig. 2*a-d*. Figure 2*a* shows thin and thick regions of fibres and Fig. 2*b-d* shows progression of the thick region and branching. The branches arise from thick regions, are thinner than the parent fibre and the parent is thinner distal to branching, suggesting that branching is the splitting of a single fibre from a parent fibre composed of many single fibres. The new branch of Fig. 2*d* grows away from the thin region at the top of the figure, suggesting that it represents a true nucleation and not a rebranching of existing fibres that may have coalesced outside the visible field. Thickening along a fibre progresses at a rate that is about the same as the growth rate of individual thin fibres, consistent with thick fibres being multiples of single fibres. This mechanism of fibre initiation is the heterogeneous nucleation of the double nucleation model^{5,19}. It is associated with three steps: initiation of a new fibre on the surface of a pre-existing fibre, growth of the new fibre alongside the old one, and branching.

Figure 2*e-f* shows centres radiating many fibres. The radial symmetry suggests they are the spherulitic domains seen in polarizing microscopy^{5,6} and ascribed to single homogeneous nucleating events. All fibres on the slide could be traced towards a centre of this kind or to a branch point. No fibres with two free ends were seen. Fibre direction (and alignment) is

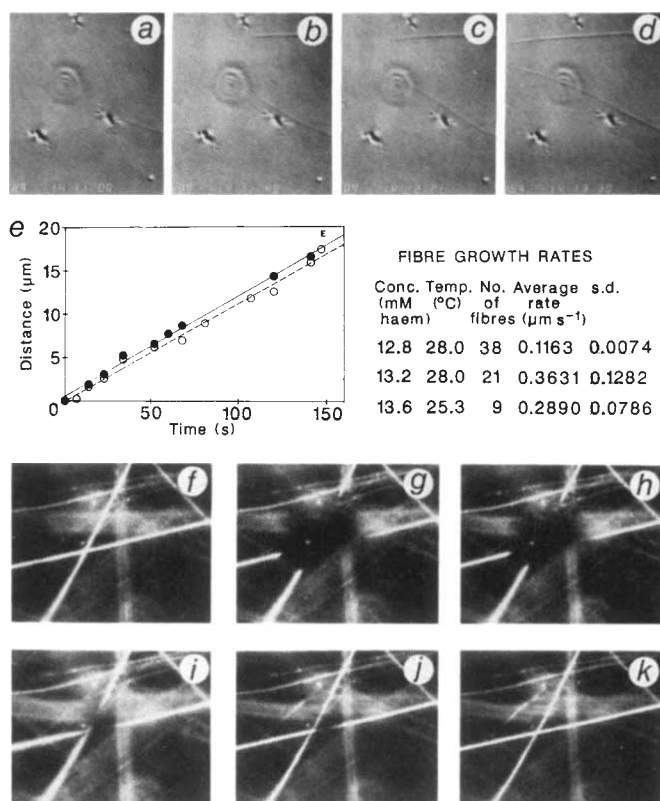
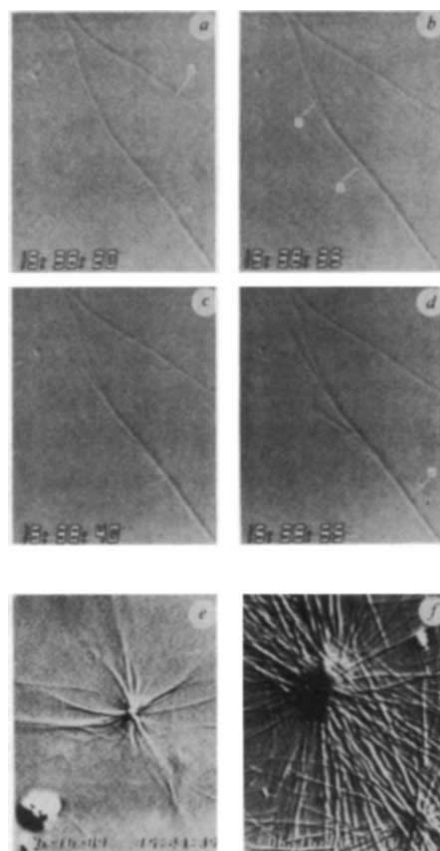


FIG. 1 Fibre growth. Serial photographs of growing fibres of deoxyhaemoglobin S at 12.8 mM (haem) and 28 °C as shown by video-enhanced DIC microscopy. The first fibres were seen 102 min after the temperature jump. *a* is at 166 min, followed by *b*, *c* and *d*, 45, 81 and 150 s later, respectively. Growth rates determined from serial video frames were 0.116 and 0.113 $\mu\text{m s}^{-1}$ respectively for the upper and lower fibres. Fibre elongation is plotted in *e* (upper fibre: closed circles, solid regression line; lower fibre: open circles, dashed regression) and the adjoining table summarizes rates. These fibres were attached to the slide surface. But many fibres were also seen oscillating with brownian motion in solution. *f-k*, Growth of cut ends of bundles of fibres in the same preparation by video-enhanced dark-field microscopy²⁰. *f*, Fibre bundles before cutting by irradiation for 15 s with a focused ultraviolet beam. *g-k*, 1, 10, 31, 40 and 51 s after the cut was completed, respectively. The two ends of each thick fibre bundle grow at about the same rate. Conc., concentration; Temp., temperature; s.d., standard deviation.

METHODS. The full width of the microscopic field was usually 40 μm . Approximate widths of the reproduced portions were 30 μm (*a-d* and *f-k*). Video DIC microscopy was as described²⁴. Specimens were 6–10 μm thick. Haemoglobin S was purified as described²⁵, dialysed into 0.1 M potassium phosphate (pH 7.0), deoxygenated at 2 °C in an anaerobic glove box with sodium dithionite (final concentration 50 mM), placed on slides and sealed under coverslips with Glyptal paint (Cenco). Slides were kept on ice until used, when polymerization was initiated by a temperature jump. Completeness of deoxygenation and the absence of methaemoglobin were confirmed spectrophotometrically. Temperatures were measured within ± 0.2 °C with a thermistor; slight drifts during experiments increased the maximum error to ± 0.5 °C.

FIG. 2 Nucleation. *a-d*, Serial frames over 35 s showing thick and thin fibre regions and branching (only fibres orientated in the same direction can be compared for thickness because contrast depends on the relation of the fibre direction to optical shear resulting from the orientation of the Wollaston prisms). Haemoglobin was about 13.8 mM (haem) at 21 °C with the first fibre seen at 12 min and this series at 25 min after the temperature jump. In *a*, the upper fibre (S) is thin and grew at $0.091 \mu\text{m s}^{-1}$. The lower fibre (seen growing before these frames) rate was $0.090 \mu\text{m s}^{-1}$. The lower fibre is thick at the lower right and thinner above. In *b*, two branches (B) occur and grow in *c* and *d*, with rates for the upper and lower 0.074 and $0.083 \mu\text{m s}^{-1}$ respectively. The branches arise from thickened regions and consist of separation of one fibre from a multiple fibre parent. *d*, Branch in the opposite direction and evidence of *de novo* nucleation within the visible field. The rate of progression of thickening for the region giving rise to the upper left branch was approximately $0.12 \mu\text{m s}^{-1}$. Picture widths in *a-d*, 17 μm . In another preparation, *e* shows fibres radiating from a single centre and *f* shows two more such structures with more radiations. These structures are relatively rare and appear to correspond to the homogeneous nuclei of the double nucleation model, although they may not be truly homogeneous in origin. On this slide at relatively high concentration (13.8 mM) and about 25 °C many fibres were seen 2 min after the temperature jump and the centres were more numerous than on slides at lower concentration. Picture widths in *e-f*, 35 μm .



determined largely from fibre origin at a radiating centre or as a branch rather than by later rearrangement. The glass surfaces fix orientation and contribute to alignment.

The radiating centres were all fixed to a glass surface, either because they arose heterogeneously on the surface or, if homogeneously nucleated in bulk solution, because fibres soon encountered a surface in the thin sample. Hence, the results do

not distinguish the nature of the nucleation. The small central structure giving rise to many fibres suggests a common nucleating site. In the double nucleation model, a single homogeneous event is followed by heterogeneous nucleation on the initial and subsequent fibres. This might be expected to create a structure in which branching rather than symmetry around a small central core predominates. Nucleating centres also formed near air

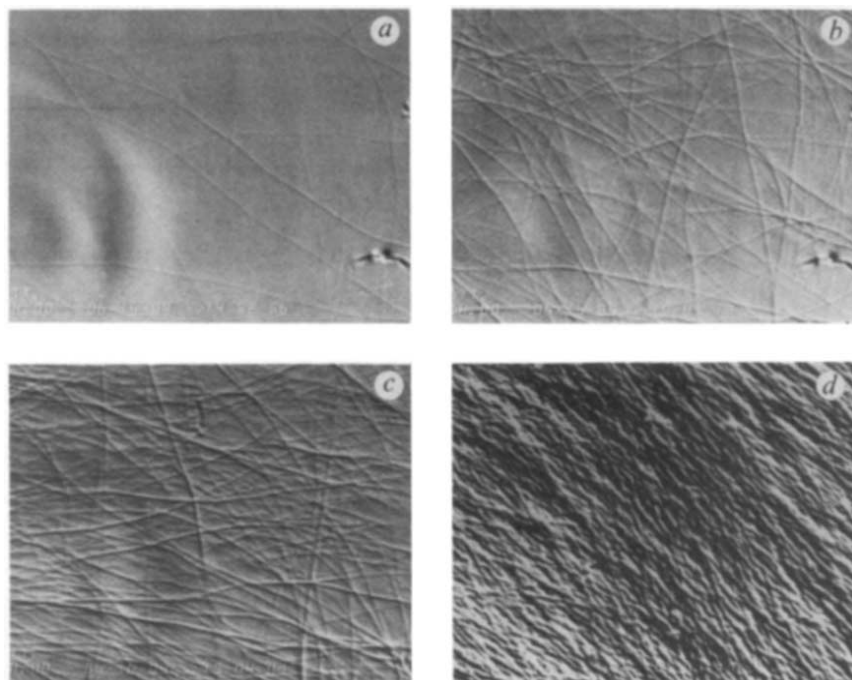


FIG. 3 The development of haemoglobin S gels. All parts are from the same slide, containing 13.4 mM (haem) haemoglobin S at about 25 °C. The first fibres on the slide were seen 7 min after the temperature jump. *a*, Gel with a few fibres 48 min after the temperature jump; *b*, same region 8 min later, over the course of which the network became much denser. At this time some parts of the slide were still devoid of fibres. *c* and *d*, Different regions slightly later (3 and 6 min after *b*, respectively) and illustrating greater fibre densities up to the extremely dense gel in *d*. Picture widths, 40 μm .

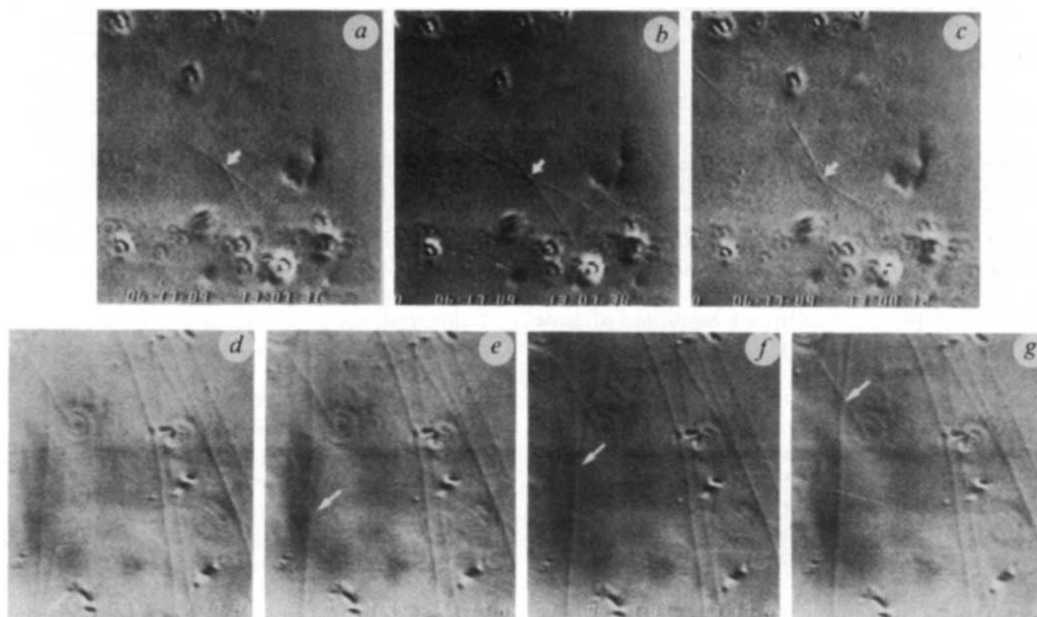


FIG. 4 Cross-linking. The existence of fixed junctions and the dynamic nature of haemoglobin S gels is shown in *a-c*. A stable junction of two fibres (arrows) remains in focus and is a cross-link in a true gel network. The fibres leading to the junction are in focus in some regions in some pictures, whereas those same regions are out of focus in other pictures. This (which can be seen in real time and much more clearly on the videotape from which this sequence is taken) demonstrates the oscillation of the fibres, their

flexibility and thus brownian motion (much larger movements and bending of fibres occur for unanchored fibre ends which are not fixed to the slide or coverslip). Picture widths: *a-c*, 25 μm . *d-g*. Taken from the same slide; side-to-side coalescence of two fibres (at the left) by zippering to form a single thick fibre. Arrows indicate the moving site of the zipper. The sequence covers 5 s and *e* and *f* are separated by less than a second. (Same slide as Fig. 1). Picture widths for *d-g*, 30 μm .

bubbles. Hence a surface or a foreign body may predispose to the initiation of gelation.

We could also induce polymerization by pressing on the coverslip. This procedure induces shear, which accelerates gelation^{18,21,22}. In preparations at low concentration that failed to polymerize spontaneously after a long period, pressing produced large amounts of polymer quickly, suggesting that shear acts by enhancing nucleation.

Figure 3 shows the development of haemoglobin S gels. Fibre density increased with time in a given field but was always highly variable over different regions of a slide.

Figure 4*a-c* shows a stable junction of two fibres and brownian motion of fibre segments. The junction is a cross-link in a network, consistent with a gelled structure and the observed

solid-like rheological properties^{7,8}. The system is neither a solution of independent fibres nor an entanglement.

Cross-links in the gel can be (1) X-shaped junctions (Fig. 4*a-c*), (2) Y-shaped branches of heterogeneous nucleation (Fig. 2*a-d*) or (3) side-to-side 'zippering' (Fig. 4*d-g*). The gel is dynamic in that fibres grow, are flexible and show brownian motion, and cross-links form continuously.

Vaso-occlusive crises in sickle cell disease depend on delay time and hence on nucleation rates²³ and on gel rheology and shear^{7,18,22}. Our findings on gel structure and growth, and on nucleating effects of shear and surfaces, may therefore be relevant to our understanding of the pathogenesis of sickle cell disease. □

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Formation of a stable triplex from a single DNA strand

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HOMOPURINE·homopyrimidine DNA sequences have been shown to form triple-stranded structures readily under appropriate conditions^{1–5}. Interest in DNA triplexes arises from potential applications of intermolecular triplexes as antisense inhibitors of gene expression^{6–8} and from the possibility that intramolecular triplexes may have a role in gene expression and recombination¹. We recently presented NMR evidence for triplex formation from the DNA oligonucleotides d(GA)₄ and d(TC)₄, which showed unambiguously that the second pyrimidine strand is Hoogsteen base paired and the cytosines are protonated at N3 as required^{9,10}. To obtain a more well defined triplex, and to provide a model for *in vivo* triplex structures, we have designed and synthesized a 28-base DNA oligomer with a sequence that could potentially fold to form a triplex containing both T·A·T and C+·G·C triplets. Our NMR results indicate that the conformation at pH 5.5 is an intramolecular triplex and that a significant amount of triplex remains even at pH 8.0.

Intramolecular triplex formation, in which half of a homopurine·homopyrimidine duplex folds back on itself to form a triplex containing two pyrimidine and one purine strand with the second pyrimidine strand Hoogsteen¹¹ base-paired in the major groove and the excess purine strand looped out as single strand, has been proposed for the unusual structure which homopurine·homopyrimidine sequences may adopt *in vivo*¹². This structure accounts for the pH and superhelical density dependent S1 nuclease sensitivity observed for such sequences^{1,13–15} and has been supported by a wide range of chemical and enzymatic studies^{1,16–21}.

The sequence of the DNA (denoted HD28) is shown in Fig. 1 as a single strand, with the potential folding pathway indicated. Spectra of the imino and C amino resonances of HD28 in 100 mM NaCl and 5 mM MgCl₂ as a function of pH at 1 °C are shown in Fig. 2. As Hoogsteen base pairing of C requires protonation at N3, and our previous study of d(GA)₄+d(TC)₄ showed triplex formation at pH < 6.1 (refs 9, 10), we initially studied the molecule at pH 5.5. At this pH (Fig. 2a), 13 hydrogen-bonded imino resonances are observed, as well as resonances from non-hydrogen bonded T iminos. In addition, resonances in the region between 10.3 and 9 p.p.m., characteristic of aminos on protonated dCs (refs 9, 10, 22), are observed. Two-dimensional NMR experiments on the sample at low pH in H₂O (discussed below) and D₂O (not shown) confirmed that the structure formed is indeed a triplex. Assignments of the imino and C amino resonances in the triplex are indicated on the figure and are discussed below (Figs 3 and 4). As the pH is raised, the spectra of HD28 do not change substantially until pH 7.0. At this pH a second set of resonances appear, which are roughly equal in intensity to those of the triplex. These resonances increase in intensity relative to the triplex resonances as the pH is raised further. At pH 8.0, four A·T and three G·C imino resonances are observed, as would be expected for the partial duplex structure shown in Fig. 1b. Although the amount of triplex, as judged by the relative intensities of triplex versus duplex imino resonances, greatly decreases above pH 6.5, it is important to note that there is still a significant amount of the triplex present even at pH 8.0. Both imino and C⁺ amino resonances from the triplex are still observable at this pH.

Although the one-dimensional spectra of HD28 as a function of pH are strongly indicative of a structural change from a duplex containing four A·T and three G·C base pairs at high pH to a triplex at low pH, our conclusion that the low pH structure is indeed an intramolecular triplex, and the assignments shown, are unambiguously based on the results of two-dimensional NMR experiments. A NOESY²³ spectrum of HD28 at pH 5.76, showing the region containing the imino proton resonances, is given in Fig. 3b. In B form DNA, imino resonances along the helix are within about 3.5 Å of each other and therefore show sequential NOE connectivities which can be used to assign the resonances^{24,25}. For triplex DNA, two sets of imino proton sequential connectivities are expected, one set between the Watson-Crick base-paired imino proton resonances and the other set between the Hoogsteen base-paired imino proton resonances^{9,10}. These sequential connectivities are observed for seven Watson-Crick base pairs and for six Hoogsteen base pairs, and are indicated on the figure. No imino proton resonance corresponding to C22 is observed.

Confirmation of the imino proton assignments and extension of these assignments to the aromatic and amino resonances was obtained from the portion of the NOESY spectrum shown in Fig. 4a and from other regions of the spectrum. Of particular interest is the observation of intra-triplet connectivities between the iminos and A amino in the T·A·T triplets. These observed triplet connectivities are consistent with the sequential assignments of the imino resonances in Fig. 3b. Each Hoogsteen base-paired T and protonated C also has a characteristic NOE to its purine H8, whereas each Watson-Crick T has an NOE to AH2 (refs 9, 10). Identification of these H8 and H2 resonances in the H₂O spectra can then be used as the basis for assignments and three-dimensional structure determination from spectra taken in D₂O.

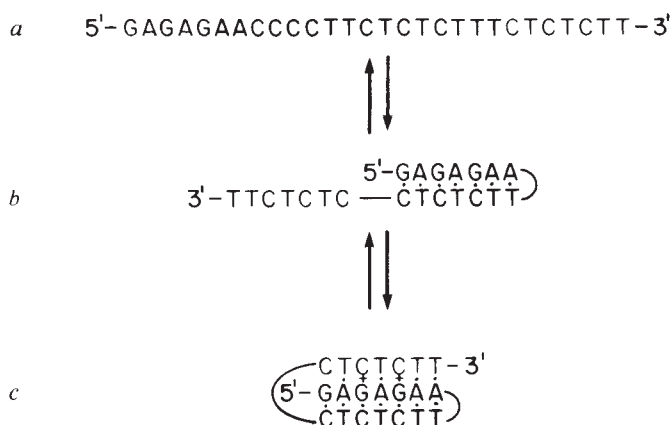


FIG. 1 Sequence of the 28-base single-stranded DNA (HD28) (a) and proposed folding pathway to form an intramolecular duplex (b) and intramolecular triplex (c). The molecule was designed so that the seven purines at the 5' end would form a hairpin duplex with the central pyrimidine tract, and the pyrimidines at the 3' end would fold over to form the Hoogsteen base-paired third strand, parallel to the purine tract. The hairpin portions of the molecule would have a loop containing four C residues connecting the Watson-Crick base pairs and a loop containing a minimum of three T residues linking the Watson-Crick and Hoogsteen base-paired pyrimidines. We note that an alternative design for the single strand triplex could begin with the pyrimidines at the 5' end to form a 5'-pyrimidine-loop-pyrimidine-loop-purine sequence. The DNA was synthesized on an Applied Biosystems 381A DNA synthesizer using β -cyanoethyl phosphoramidites and 1 μ mol columns. Three to five syntheses were combined and purified by gel filtration following the method of Kintanar *et al.*³¹. Aliquots of the peak (A_{260}) column fractions were kinased with [³²P]ATP and run on sequencing gels, and only fractions containing only one full-length band were pooled. Pooled fractions were lyophilized and stored in a desiccator until used. NMR samples were prepared by dissolving 3–8 mg DNA in the appropriate salts and water and adjusting the pH with NaOH. Samples were transferred to an NMR tube, dried in the tube by a stream of N_{2(g)}, and redissolved in 400 μ l 90% H₂O/10% D₂O or in 99.996% D₂O.

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The data presented here establish that HD28 folds, under appropriate conditions, to form an intramolecular triplex with seven Watson-Crick and six Hoogsteen base pairs. The Watson-Crick strands are connected by a 4C loop. A loop size of four to five bases is optimal for B-DNA hairpins²⁶⁻²⁸ and, therefore, may facilitate initial formation of the partial duplex. The second

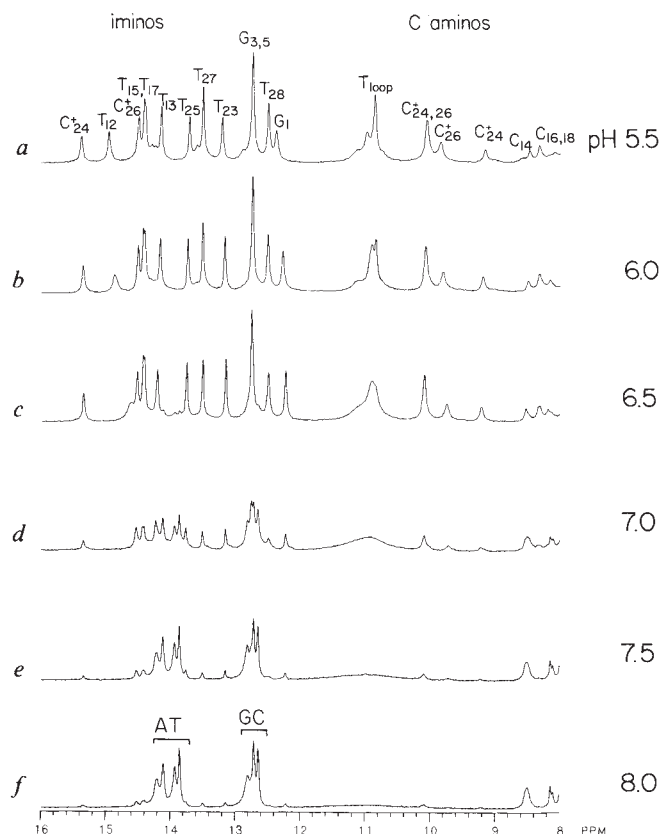


FIG. 2 Spectra of the imino and C amino resonances of HD28 in 90% H_2O /10% D_2O , 100 mM NaCl, 5 mM $MgCl_2$, 0.8 mM strand, 1 $^{\circ}C$, as a function of pH: a, pH 5.5; b, pH 6.0; c, pH 6.5; d, pH 7.0; e, pH 7.5; and f, pH 8.0. The sample was initially prepared at pH 5.5, and the pH was subsequently adjusted for each spectrum by addition of appropriate amounts of 0.01–0.05 M NaOH (meter reading, in the NMR tube). After obtaining the spectrum at pH 8.0, the pH of the sample was lowered to pH 5.5; the spectrum obtained was identical to a (not shown). Some changes in the spectra of the triplex are observed between pH 5.5 and pH 6.5, predominantly broadening and upfield shifting of the T12 imino resonance and broadening of the imino resonances from the T residues in the loop. These changes are the result of an increase in exchange rate of these non-hydrogen bonded and terminal imino resonances as the pH is raised³² rather than a significant change in the structure of the triplex. Some additional resonances, which do not correspond to resonances in the duplex, are also observed at the lower pHs, for example between T17 and T12, between T25 and T27, and underneath T27. We attribute these to a small amount of some alternative structure which we have not yet characterized. Resonances from the duplex begin to be evident at pH 6.5. We note that an alternative duplex structure, in which the first 18 bases of two HD28 molecules base pair to form a duplex with a central four-base bulge, cannot be rigorously excluded as the structure at pH 8.0. But such a structure seems unlikely on the basis of previous NMR studies of DNA hairpin versus bulge structures²⁷, and judged by the fact that the linewidths of the imino resonances for this structure are comparable to those of the triplex, which we would not expect if the molecule was a dimer. We can also rule out any significant amount of interstrand triplex formation (pyrimidine strand of one oligonucleotide binding to the hairpin Watson-Crick purine strand of another), based on intensities of the non-hydrogen-bonded T iminos at low pH, NOESY spectra in D_2O , and observed linewidths. Spectra were acquired using a 11° spin echo pulse sequence³³ ($90^{\circ}_x - \tau - 90^{\circ}_x - \Delta_1 - 90^{\circ}_y - 2\tau - 90^{\circ}_y - \Delta_2$) with the excitation maximum at 12.5 p.p.m. The spectral parameters were 8K complex points, spectral width 12,048 Hz, recycle delay 2 s, and 256 acquisitions. The spectra were line broadened by 3 Hz before Fourier transformation.

loop connecting the Watson-Crick and Hoogsteen base-paired pyrimidines is of particular interest because it should correspond roughly in structure to the loop in intramolecular triplexes that may be present *in vivo*²¹. This loop is composed of three T residues plus C22, which potentially could base pair with G1 but apparently does not. Thus, our data indicate that the minimum loop size for an intramolecular triplex is four bases. Models of intramolecular triplexes by Dahlberg and co-workers, based on chemical protection studies, show a five-base loop²¹. On the basis of our data, this seems to be a reasonable model, because the 5' end in our triplex would extend out of the loop in the 3' direction in an *in vivo* triplex, thus possibly disrupting the G1–C18 base pair. The pH studies on our single-strand triplex also show that in intramolecular triplexes in which half

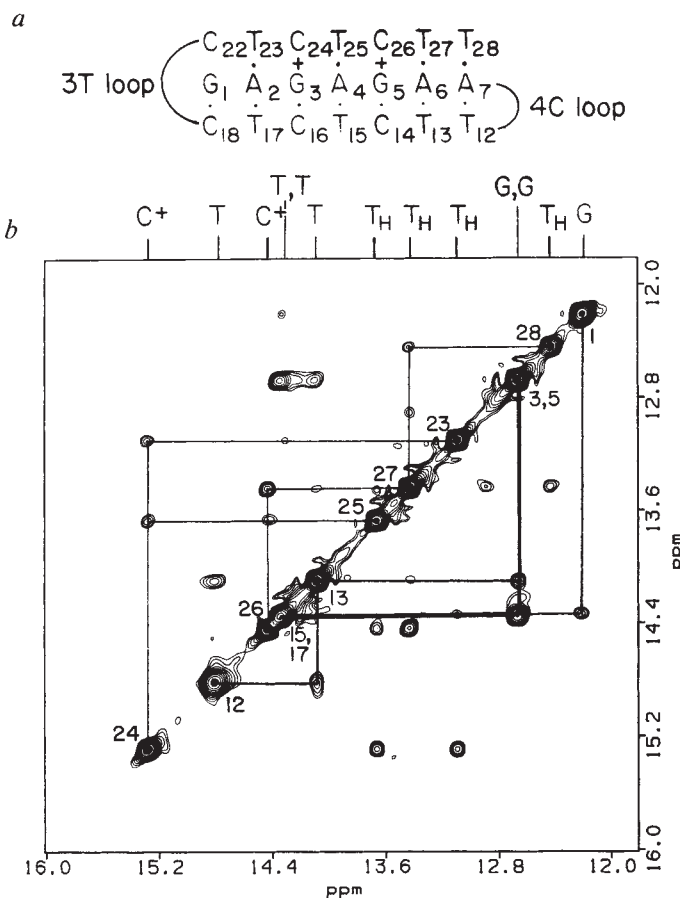


FIG. 3 a, Folded triplex with the numbering system. b, Portion of a NOESY spectrum of HD28 in 90% H_2O /10% D_2O at 1 $^{\circ}C$ showing the region containing the imino proton resonances. Sample is 100 mM NaCl, 5 mM $MgCl_2$, pH 5.76, 2.7 mM strand in 400 μ l. Assignments to proton type are indicated on the top of the spectrum: C⁺ (Hoogsteen base-paired protonated C), T (Watson-Crick base-paired T), T_H (Hoogsteen base-paired T), G (Watson-Crick base-paired G). Sequential connectivities between the Watson-Crick base pairs are indicated at lower right and those between the Hoogsteen base pairs are indicated at upper left. The spectrum was obtained in the pure absorption mode following the method of States, *et al.*³⁴ using the standard $90^{\circ} - t_1 - 90^{\circ} - \tau_m - 90^{\circ} - \text{acquire}$ pulse sequence²³ except the last 90° pulse was replaced with a 11° spin echo pulse sequence and phase cycled appropriately to suppress the large water resonance³³. The carrier was centered at the water resonance and the delay τ was adjusted so that the maximum excitation was at 12.5 p.p.m. The acquisition times in t_1 and t_2 , respectively, were 170 and 24.9 ms, 2,048 complex points in t_2 , and 300 t_1 increments were obtained. The sweep width was 12,048 Hz in both dimensions and 64 scans were acquired per t_1 value. The mixing time (τ_m) was 100 ms with a homospoil pulse on during the first 30 ms and the recycle delay was 1.9 s. The spectrum was zero-filled in t_1 to give a final $2K \times 2K$ real data matrix and spectra were apodized by a 50° phase shifted squared sinebell in both dimensions. A fifth-order polynomial baseline fitting was applied in the f_2 dimension. Spectra were processed with the software program FTNMR (D. Hare).

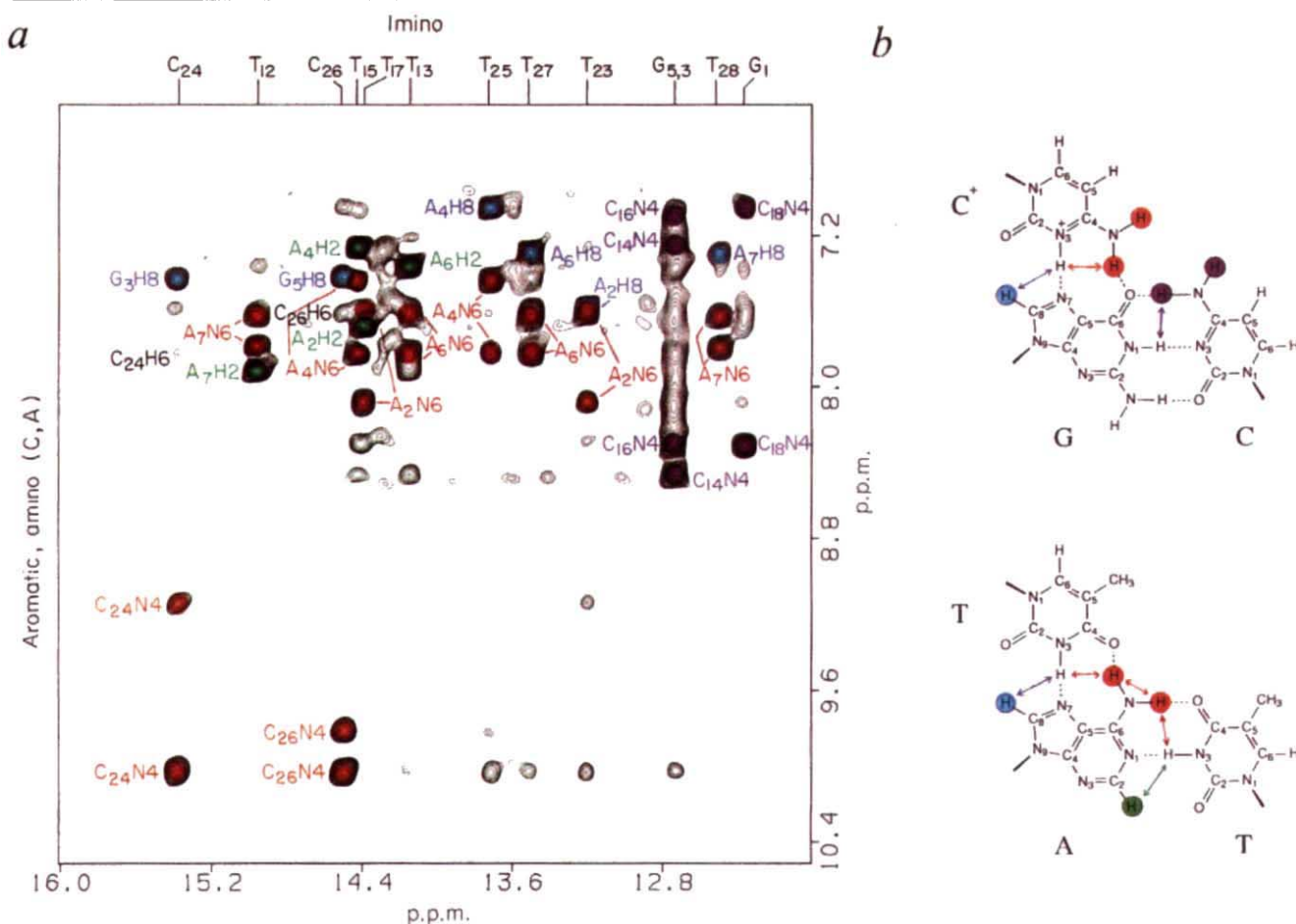


FIG. 4 a Portion of NOESY spectrum of HD28 in 90% H_2O /10% D_2O at 1 $^\circ\text{C}$ showing the region of cross peaks between the imino and the aromatic and C and A amino resonances. Sample and spectrum are the same as Fig. 3. Intratriplet NOEs between the imino and the H₈, H₂, AN₆ (A amino), CN₆ (C and C⁺ amino) cross peaks are labelled. The lower field C and C⁺ amino resonance in each pair is the hydrogen bonded one. The C⁺ iminos were clearly identified by their strong cross peaks to the lowfield shifted C⁺ amino resonances¹⁰. The two amino resonances in each C⁺ base were identified as pairs by their cross peaks to each other in the amino, aromatic region of the spectrum (not shown). The pairs of A amino and C amino resonances were identified in the same way. Only two sets of C⁺ amino

resonances are observed. The Hoogsteen base-paired T iminos which are identified by sequential connectivities in Fig. 3b also have cross peaks to the neighbouring C⁺ aminos. Examination of the base triplets shown in b shows that the A amino protons in each T-A-T triplet should show NOE connectivities to both the Watson-Crick and the Hoogsteen base-paired T iminos, for example, T17 and T23 should both have cross peaks to A2 aminos. b, C⁺-G-C and T-A-T triplets. Intratriplet NOE connectivities to the imino protons are denoted by coloured circles: imino-H₈ (blue), imino-AH₂ (green), C⁺ imino-amino (orange), imino-C amino (purple), and imino-A amino (red).

of the Hoogsteen paired bases are C residues, a significant amount (~50%) of triplex is present at neutral pH even though protonation of C is required. As supercoiling *in vivo* may stabilize this structure even more (as is the case for Z-DNA²⁹), the pH dependence of this structure does not obviate its potential presence *in vivo* at neutral pH.

Finally, we note that the single-strand triplex provides a simple model system for studying the sequence specificity of triplex formation, as one or more changes in the sequence and composition of the base triplets can easily be incorporated into the DNA oligonucleotide. Such studies are in progress to evaluate the base-pairing scheme for the G-T-A triplet within a pyrimidine-purine-pyrimidine motif proposed by Dervan and co-workers³⁰ and the G-G-C triplet within a purine-purine-pyrimidine motif proposed by Hogan and co-workers⁶. The results of these studies should be useful in designing sequences for antisense inhibitors of gene expression. □

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Atomic force microscopy

C. B. Prater, H. J. Butt and P. K. Hansma

The atomic force microscope can resolve individual atoms and molecules on some sample surfaces — even nonconducting samples in water or other fluids.

THE atomic force microscope (AFM)¹ is a member of the growing family of scanning probe microscopes². The AFM can image the surface of a variety of materials including biological samples in physiological environments. Commercial AFMs (Digital Instruments, Santa Barbara, California; Park Scientific Instruments, Mountain View, California) (Fig. 1) have just



FIG. 1 An atomic force microscope prototype (Digital Instruments, Santa Barbara, California) imaging a 'living' plant leaf.

become available and can be used in applications from surface science to quality control and from tribology to biology.

How it works

The AFM (see Fig. 2) creates topographic images of surfaces by scanning a sample under a sharp stylus. The stylus is attached to a cantilever, which is deflected as the stylus interacts with the surface. The stylus may be in direct contact with the surface or it may be vibrated above the surface. For high-resolution imaging and most routine topographic profiling, the stylus is kept in direct contact with the surface. The non-contact method has been used to image magnetic and electric fields^{3,4}, and liquid films⁵.

Images are produced by measuring the deflection of the cantilever as the sample is scanned under the stylus on an x,y,z piezoelectric translator. AFMs are available commercially with large scan ranges of up to 75 μm . AFMs with a 25- μm range have zoomed in to resolve individual atoms, giving a range of magnification from 3×10^3 to 10^8 .

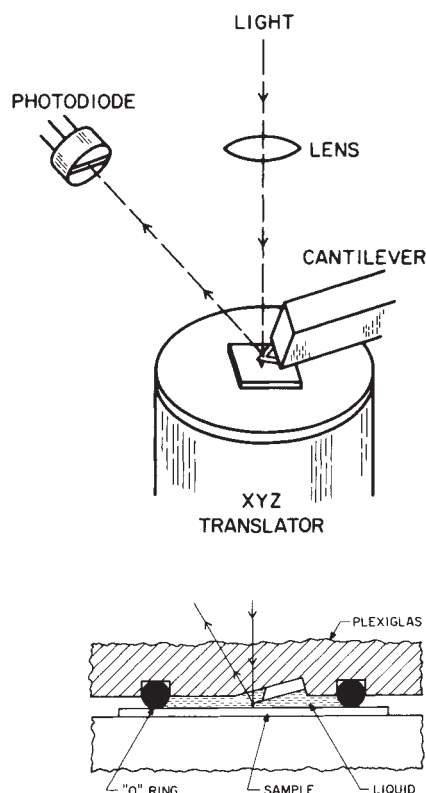


FIG. 2 Schematic diagram of the atomic force microscope.

The most common techniques for measuring the deflection are (1) measuring the phase of laser light reflected off the back of the cantilever (an interferometry technique)³ and (2) measuring the deflection of a laser beam that bounces off the back of the cantilever (the optical-lever technique)^{6,7}.

Commercially available AFMs combine the optical-lever technique with microfabricated cantilevers⁸. The cantilever deflection is detected as follows: a sharp, pyramid-shaped stylus, which has been microfabricated onto the tip of the cantilever (Park Scientific Instruments), is allowed to touch gently the sample surface. A surface feature deflects the cantilever through atomic repulsion by a distance δz . This deflection causes a laser beam reflecting off the cantilever to deflect by an angle of roughly $2\delta z/l$, where l is the length of the cantilever, usually 100–200 μm . This deflection of the reflected beam is seen as a change in the difference between light striking the segments of a two-segment photodiode. Because of the optical lever, the deflection of the laser beam at the photodiode is

roughly 1,000 times the deflection of the cantilever.

The microscope can be operated by simply measuring the cantilever deflection or, more often, by using a feedback system to keep the deflection constant (constant-force mode). In constant-force mode, the feedback loop can keep the force of the stylus on the sample lower than 10^{-9} N for samples in water, or roughly 10^{-7} N in air. Imaging with the sample in liquid eliminates meniscus forces that can pull the stylus destructively into the sample, and thus makes it possible to use smaller forces.

AFM applications

As the AFM can image conductors and nonconductors, and can image samples in air, liquids or vacuum, it is finding a wide range of applications. The AFM can routinely resolve atomic structure on hard minerals, and even on soft metals like gold⁹. It has been possible to resolve individual molecules adsorbed onto a zeolite crystal surface¹⁰. Because the AFM can resolve individual atoms in a variety of environments, it will become an important tool in such fields as geology, surface science and electrochemistry.

The AFM is also a useful tool for biological research because samples may be imaged in physiological buffers. This can simplify sample preparation as samples may not need to be fixed, stained, dried or metal-coated. Figure 3 shows an AFM image (8- μm scan) of purple membranes. These membranes, roughly 0.5–1 μm across and 5-nm thick, form part of the cell membrane of *Halobacterium halobium*. The membranes shown here were bound to glass slides that were silanized with aminopropyltriethoxysilane. The image was obtained under the aqueous buffer

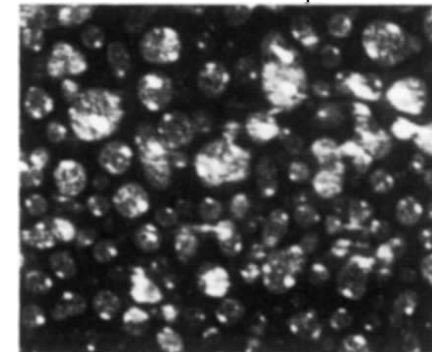


FIG. 3 Purple membranes in aqueous buffer as imaged with the atomic force microscope. The scan area is 8 $\times$ 8 μm .

1 mM CaCl_2 , 10 mM KCl, 5 mM Tris, pH 7.2. At higher magnification, it is possible to resolve the individual bacteriorhodopsin molecules that make up the membrane^{11,12}. The AFM has also been used to image crystallized lipid bilayers¹³ and amino acid polymers¹⁴ with 0.5-nm resolution.

The AFM obtains images at a fast enough speed (typically, 1–30 seconds) that it can be used to observe some biological and chemical processes. In one experiment, we were able to observe the polymerization of fibrin, a major component in blood clotting, at almost molecular resolution¹⁴.

Additional scientific applications are being investigated in fields from geology to surface science and electrochemistry. So far, technological applications for the AFM include imaging integrated circuit chips, optical and X-ray components, data storage media and other critical surfaces.

Although the AFM can resolve atoms on hard surfaces, so far, it has revealed details only as small as 10–20 nm on the surface of whole cells. A major challenge for researchers is to develop new sample preparation techniques that will allow smaller details to be imaged. The sample must both be held firmly to a substrate and must be rigid enough not to be damaged or distorted by the applied force of the stylus. For this, AFM users may benefit from preparation techniques developed for other forms of microscopy, while enjoying the AFM's ability to image samples in diverse environments.

In conclusion, the AFM can image the surface of almost anything in gases, liquids or vacuum, and with atomic resolution on rigid samples and scan areas of many microns. However, there are still many challenges ahead, including improving sample preparation techniques in order routinely to achieve high resolution. □

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In the picture

Micro '90, the international showcase of instruments for microscopy to be held next week in London, will feature a video printer for print, slide or overhead transparency production, and a field emission source scanning TEM.

POLAROID's QuickPrint VI-350 (Freeze-Frame Multi-Scan) **portable multi-scanning video printer** is designed to produce high-quality prints or 35-mm slides directly from an imaging system or a PC (*Reader Service No. 101*). At £2,500 (UK), QuickPrint incorporates a high-resolution multi-scanning CRT so it can be used with a broad range of RGB analogue signals in the 15–35 kHz sweep frequency range.



Polaroid's portable video printer for slides, prints and mini-overheads.

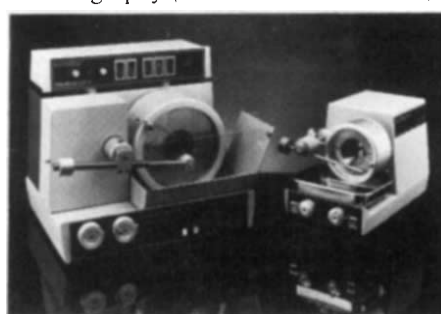
Consequently, the video printer can be jugged up to conventional light and confocal laser scanning microscopes, image analysis equipment and with most PCs equipped with a CGA, EGA or VGA graphics card. QuickPrint is useful for any application requiring black and white and colour AutoFilm prints, as well as instant and conventional 35 mm black and white and colour slides, and mini-overhead transparencies. In order to provide optimum image quality, the user has full control of levels of red, green and blue, as well as brightness and contrast. In addition, QuickPrint has a double-exposure option for printing images from two different sources on the same photo, and a 'fill' function, which fills the gaps between field scanning lines to give a smoother image. Polaroid's new product line will be on show on stand 30.

Kontron Electronics, exhibiting on stand 49, will be launching its fastest and most comprehensive **image analyser** at Micro '90 (*Reader Service No. 102*). Based on the AT-compatible platform, the new IBAS can analyse images up to ten-times faster than previous models, says Kontron. Both hard disk and image storage can be as high as one and two gigabytes, respectively.

Sample preparation

On stand 23, Malvern Instruments will be showing off a new range of **precision**

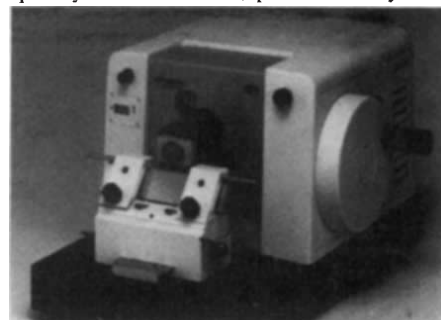
cutting and polishing machines — sample preparation systems for microscopy and metallography (*Reader Service No. 103*).



Cut and polish samples with precision tools from Malvern.

Malvern's Microslice diamond saws are suitable for cutting hard, soft or brittle materials: the delicate and controlled cutting action minimizes surface damage to the specimen, says Malvern. Both peripheral and annular blades are available, and the rigidity and thinness of the latter allows fine cuts to be made with minimum wastage. The Microslice 2 can make cuts up to 50 mm in depth while, for larger samples, Malvern recommends the Microslice 4, which is available with either manual or automatic indexing for repetitive slicing operations. For finishing specimen surfaces to a high degree of flatness and parallelism, Malvern offers the Multipol 3 polishing machine. The automatic version includes an automatic washdown facility, full user control of the timing of each operation and storage of more than 200 polishing routines.

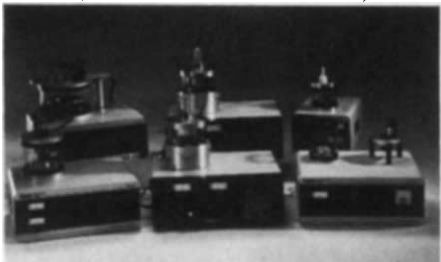
Optech Scientific Instruments is distributing the German-made Microm HM 330 routine **rotary microtome** (*Reader Service No. 104*). A special design feature includes specimen retraction on the return stroke which, Optech says, improves the quality of the section, provides easy rib-



Optech's rotary microtome — HM 330.

boning and increases the edge life of the cutting tool. An automatic trimming mode, section counter with reset and safety loading device are additional features of the Microm HM 330. To satisfy all microtome's needs, Optech offers a basic HM 320 model, which has all the features of the HM 330, but without the retraction feature; the HM 340, with retraction and with a motor-driven coarse feed; the heavy-duty HM 350 model; and the HM 500 range of cryostats. Optech will be open for business on stand 56.

The microscience division of Bio-Rad has introduced a new line of **sputter and carbon coating systems** designed to allow the scanning electron microscope user to obtain high-quality results from the instrument (*Reader Service No. 105*). The

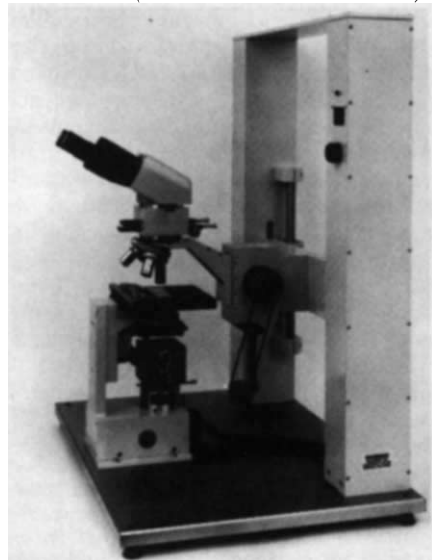


Sputter and carbon coating systems.

sputter coater line — SC500, SC502, SC510, SC515 and SC650 — includes instruments for the budget conscious, high-resolution coating systems, and models that will coat specimens up to 200 mm (8 inches) in diameter. In addition, the TB500 carbon coater is designed to coat specimens with carbon using either rod or fibre evaporation. Bio-Rad's stand at Micro '90 will be 19.

Microscope assortment

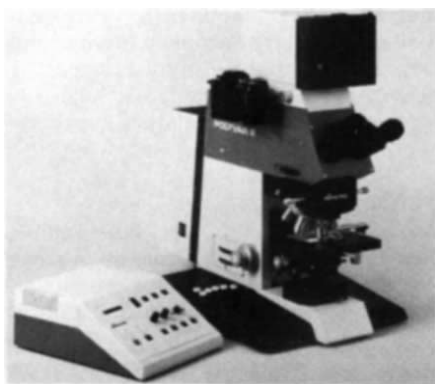
The M2A upright microscope from Micro Instruments, which is designed for **micro-manipulation studies**, can be fitted with a differential interference contrast (DIC) attachment (*Reader Service No. 106*).



M2A microscope with DIC capability.

Coarse and fine focusing adjustment are located on the head of the M2A so that the stage is at a fixed height in relation to the micromanipulators, which are fitted to the microscope's large base plate. The DIC system is, says the company, useful for distinguishing structures in thick sections and embryos, with particular applications in patch-clamp recordings in slices, transgenic mouse production and embryo research. Any microscope objective can be used with the DIC attachment. Consequently, the user can select the objective to suit the work being carried out and adjust the substage interference unit accordingly. The DIC M2A can also be fitted with an incidence fluorescence illuminator to allow particular structures to be located using the DIC system and then to switch over to incident fluorescence using the same objective. See stand 47 for Micro Instruments.

Following the merger between Cambridge Instruments and Wild Leitz, the latest research **photomicroscope** designed especially for biomedical applications, and called the Polyvar 2, can be seen on stand 16 at Micro '90 (*Reader Service No. 107*). Polyvar 2 has a new illuminating system with universal lamp housing for transmitted and incident light; this pro-



Polyvar 2 — Wild Leitz' photomicroscope.

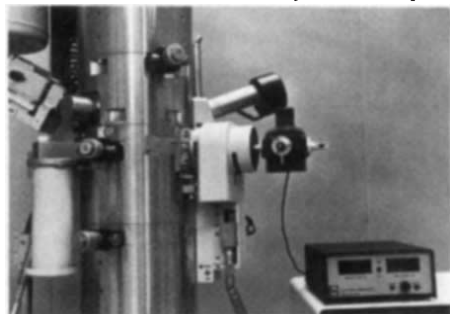
vides pre-aligned 85 W halogen reflector lamps or XBO 75-W or HBO 100-W high-intensity arc lamps for incident light fluorescence. Such a design makes it both quicker and easier for lamp replacement, says Wild Leitz. The upgraded IL-fluorescence system with triple fluorescence module and a new optical system improves results with fluorescence microscopy. Polyvar 2's design encompasses a new plan fluor apo objective series, providing, says Wild Leitz, high-resolution with all contrast methods and fluorescence. Wide-field documentation with all formats and improved colour rendition are also features of the new design. The new telematic NC intelligent control system, which combines an automatic objective device with a motorized widefield condensor, allows direct objective selection at the touch of a button with automatic setting of the condensor optics. For extended docu-

mentation purposes, the trimatic triple camera system is microprocessor controlled.

Cambridge Technology has been appointed distributor for a new low-cost K2 B10 **confocal scanning optical microscope attachment** for modern microscopes, which is made in the United States by Technical Instruments (*Reader Service No. 108*). The attachment provides real-time confocal imaging with a broad-band light source for viewing a full range of biological and industrial samples. The set-up provides five operating modes: confocal brightfield, confocal epifluorescence, brightfield epifluorescence, brightfield and brightfield incident light. See the K2 B10 on stand 2.

TEMs

Recent developments announced by Hitachi Scientific Instruments now makes it possible for the **direct observation by electron microscopy of cryogenically-prepared samples** using the company's Model H-7000 transmission electron microscope (TEM) coupled to the Gatan Model 626 cryotransfer system (*Reader Service No. 109*). The new system set-up is



Observe cryogenic samples by TEM — see Hitachi's set-up on stand 15.

designed to overcome the difficulties associated with introducing frozen specimens into the column. Initially, samples are observed at equilibrium temperatures of about -170°C . To enhance the detail of the image, a temperature-control current gradually heats the sample until the ice steadily sublimates at temperatures between -120 to -90°C . Temperature control is, says Hitachi, monitored to be accurate to $\pm 1^{\circ}\text{C}$. A cold trap and cold finger fitted in the vicinity of the specimen allow stable observation of the sample at these temperatures, unaffected by adsorption of moisture. Real-time image display is possible with the TEM's optional integrated TV camera. The instrument's ultra-zoom magnification system allows for rapid changes in magnification from 1,000 to 600,000 $\times$ and without the need for image rotation or inversion. Full computer control of the scanning attachment provides for TEM, STEM, SEM, EDX and EELS analysis at the touch of a button and without specimen repositioning. Hitachi will be on stand 15.

With the life science market in mind, Philips Analytical says its CM10 **transmission electron microscope** (TEM) can be used by both experienced and novice operators alike (*Reader Service No. 110*). The reason for this lies with the system's microcontroller, which keeps the number of microscope controls to a minimum. The microcontroller also provides the user with a continuously updated display of the instrument's status and allows parameters and conditions to be altered by means of softkeys. The use of multi-function controls further simplifies operation. The CM10's column is supplied pre-aligned, so there is no need for mechanical adjustment: any fine alignment necessary can be done electromagnetically, says Philips. The magnification range of the TEM's high-resolution, high-contrast objective



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lens is 20 to 510,000 $\times$. Optimum focus setting can be maintained over the magnification range with a choice of seven automatic contrast functions. The instrument is also fitted with a eucentric tilting goniometer and accepts a range of specimen holders to suit specific applications. For example, low-temperature microscopy is made possible by use of the cryo-specimen holder and transfer system. See the CM10 on stand 7.

VG Instruments, exhibiting on stand 4, has developed a new **field emission source scanning transmission electron microscope** — the HB501UX — for high-resolution imaging applications (*Reader Service No. 111*). The HB501UX operates at 100 kV and can produce images with a point resolution of 0.22 nm by using a special high-angle annular detector, says VG. Images contain strong atomic number contrast so that chemical changes at interfaces are visible at atomic resolution. Compared to conventional transmission electron microscopes, images produced with the HB501UX have a 35 per cent better resolution for a given acceleration voltage and objective lens. This, says VG, allows the voltage to be decreased and the specimen imaged under conditions free of radiation damage, without loss of resolution. The optical column of the instrument is designed to give maximum thermal and mechanical stability. Careful design of the new objective has enabled a high-performance X-ray microanalysis capability to be retained.

SEMs

The JSM 6100 is the latest **large-sample analytical scanning electron microscope** (SEM) to come off the Jeol production line — see stand 13 (*Reader Service No. 112*). The JSM 6100's eight-inch chamber

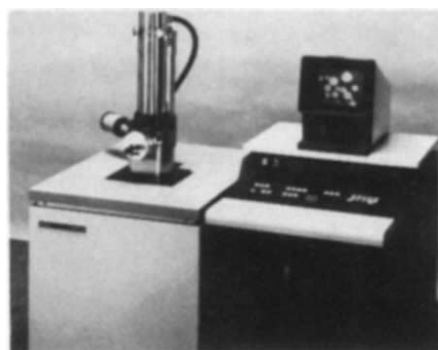


Jeol's JSM 6100 large-sample SEM — ideal for whole specimens.

allows the user to load whole clinical or biological specimens, metal samples or semiconductor wafers. A motorized stage allows movement of the specimen, including tilting and rotating through 360°. A high-resolution, built-in framestore (1,024 $\times$ 1,024) can create a stereo effect

to allow the user to measure depth within the specimen by storing and comparing images tilted at different angles. An optional light microscope can help the user line up the high-magnification SEM (10 to 300,000 $\times$) with identifiable features. Users have both manual and full keyboard control of lens parameters, acceleration voltage and alignment settings, autofocus, contrast and brightness, and astigmatism correction. Secondary and backscattered images can be displayed, stored and compared. A new image processing function allows image averaging and storage, filing of acquired still images, and comparison of two or four images displayed simultaneously on the CRT. According to Jeol, this allows the non-invasive observation of specimens, reduces 'noise' and enhances 'weak' images.

Following hard on the heels of the ISI/ABT 55 **scanning electron microscope** (SEM), ISI has introduced the ISI/ABT 32 SEM, which has full microprocessor



ISI's SEM with full microprocessor control.

control and a resolution that the company guarantees at 50Å (*Reader Service No. 113*). The standard instrument has seven accelerating voltage settings, full microanalysis mode with spot, line, line profile, X-ray image, and waveform monitor. Data displayed on the CRT include acceleration voltage, magnification, film number, working distance and spot-size factor. The SEM has CPU-controlled automatic functions for vacuum sequencing, filament saturation, gun alignment, focus, astigmatism correction, contrast and brightness selection. More experienced users can, however, override these functions. The ISI/ABT 32 is available in various configurations designed to suit user needs. The instrument is fully compatible with energy-dispersive X-ray analysis. Other configurations include a wetSEM version, a large-specimen chamber version and a model that includes an integrated image analysis system. □

These notes are compiled by Diane Gershon from information provided by the manufacturers. To obtain further details, use the reader service card bound inside the journal. Prices quoted are sometimes nominal, and apply only within the country indicated.